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Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Biologia

2017

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2017

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Hemorrhois algirus, Tarfaya, Morocco. Photo by Daniele Salvi

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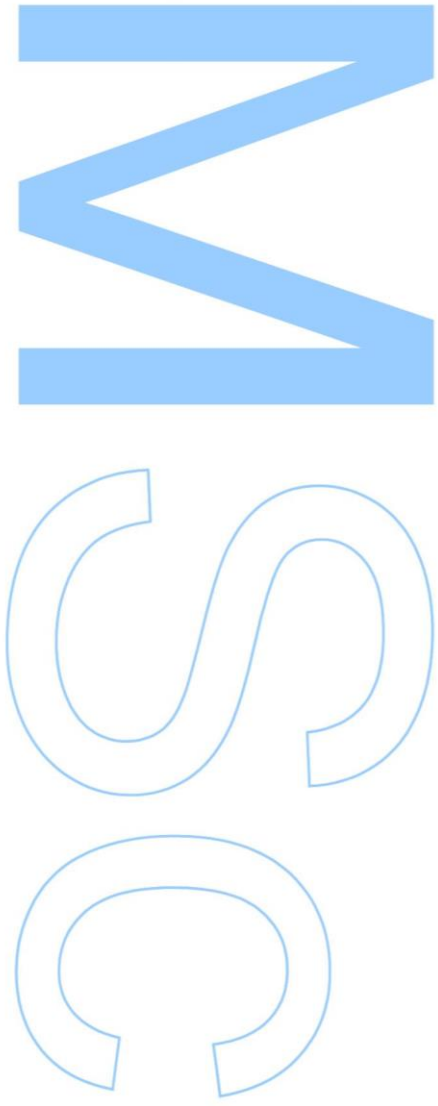
Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____



Acknowledgments/Agradecimentos

I would like to thank first to my co-supervisor, Daniele Salvi. It was you that guided me from the very beginning. Thank you for your perfectionism, for your guidance, for all the Skype meetings and, specially, for your patience in these last hard days. I am really grateful to have you answering my questions and making me comfortable to ask them. I just hopped you were here with me, in Cibio, so I could have learned even more with you.

In second, I would like to thank to my supervisor, James Harris. Thank you for fishing me in those classes, making me watch pictures from Seychelles and making me think: “I really want to do that”. Thank you for the fieldtrip and for the ten days without a shower. I would do it again. Thank you for your availability whenever I needed. You have so many stories and you obviously love what you do. One day, I hope to be like you.

I would also like to thank my other research collaborators, José Carlos Brito, Fernando Martínez-Freiría, Philippe Geniez and Gabriel Martínez del Mármol Marín for sending me so many samples and allowing me to have a great dataset.

Agradeço também ao Luís Machado, à Joana Mendes, à Margarida Henrique e à Daniela Rosado por me terem disponibilizado extrações e sequências, facilitando muito o meu trabalho de laboratório.

Queria agradecer ao pessoal do Ecolab, pela companhia de trabalho, pela música, pelas risadas e pelos conselhos e, particularmente, à Joana Santos por me guiar nos meus primeiros passos no laboratório e por me responder a todas as dúvidas que eu lhe coloquei.

Um obrigado especial ao grupo das Rendas… João, Lara, Filipa, Sophia e Margarida… e a todos os outros colegas de Mestrado que nos fizeram companhia pelo Cibio, mesmo que não fossem sempre. Obrigado pelo apoio mútuo, pelas viagens de metro, pelos jantares, pelas risadas e por todos os momentos que me deram durante estes dois anos.

Um muito obrigado aos meus colegas de Licenciatura. Obrigado por os nossos momentos únicos não terem acabado e por estarem sempre prontos para nos juntarmos outra vez. Obrigado pelas mensagens nas fitas que me fizeram chorar e sentir uma pessoa querida junto de vocês. Um dia vamos ser muito velhinhos e vamos continuar a juntarmo-nos para mais momentos. Biopóneis para sempre!

Agradeço ao meu pai, por todos os conselhos, todas as dicas, toda a preocupação e todos os abraços que sempre me acalmaram quando eu mais precisei. Tu és uma pessoa que não se desfaz dos seus princípios e que por isso sempre procurou dar tudo o que os teus filhos precisavam para crescer. E, de repente, já estamos crescidos. Um dia, espero poder dar aos meus tudo o que tu me deste.

Obrigado à minha mãe e à minha irmã. Por me estarem constantemente a perguntar como ando e se está tudo a correr bem. Por se preocuparem comigo e por me darem todo o descanso mental, o conforto e a refeições que eu precisei para acabar a tese com tempo (possível) e com calma. Não há nada como a comida de casa.

Obrigado à minha família por toda a preocupação, todos os telefonemas e todas as orações. Sendo o primo mais velho sempre senti uma responsabilidade de ser um exemplo a seguir e de deixar sempre todos orgulhosos. Espero que na vida continue a fazê-lo, mesmo quando já cá não estiverem todos os que sempre me acompanharam.

E, por fim, um obrigado muito especial à minha companheira de aventuras. Pelos cozinhados, a paciência, a companhia, os abraços… por tudo. Mais um passo que dei para, no fim, embarcar na maior aventura de todas…

Abstract

Biodiversity is not homogeneously distributed on Earth. There are regions called “hotspots” where biodiversity, much of which endemic, is more highly concentrated than in others. Many of these are areas both poorly studied and at great risk because of human impact. So, it is essential to study them to find the best way to protect them.

In North Africa, the Maghreb region is part of the Mediterranean basin hotspot. This region has high biodiversity richness as it encompasses Mediterranean habitats, a super-arid desert, the Sahara, and mountain habitats, in the Atlas mountain chain. The dynamics of the climate and habitat distribution through the last million years has shaped the genetic diversity of the species found in this region, something recent molecular assessments have revealed. Nevertheless, the biodiversity of this region is still underestimated due to political instability and the difficult access to many remote areas.

The Algerian whip snake, *Hemorrhois algirus*, and the horseshoe whip snake, *Hemorrhois hippocrepis*, are the two species of their genus inhabiting this region. The two have mostly an allopatric distribution, with the first one being present in southern semi-desert regions and the second inhabiting northern Mediterranean habitats. Despite having distinctive phenotypic characteristics, many individuals have been found with intermediate traits in regions of sympatry, in Morocco. Former genetic studies of these species have been mostly focused in their phylogenetic relationships. However, *H. hippocrepis* has been the focus of two phylogeographic assessments, one with mitochondrial and the other with nuclear gene fragments, that uncovered little genetic diversity. On the other hand, there is no data on the intraspecific variation of *H. algirus* and the nature of the intermediate forms between the two species is not clear. The main objectives of this work were: (1) to assess the phylogeographic patterns of *H. algirus*, (2) to assess the phylogenetic relationship between the pure and intermediate forms of *H. algirus* and *H. hippocrepis* and (3) to relate these results with the current intraspecific taxonomy of the species.

To do this, a total of 65 *H. algirus* samples from most of its range and *H. hippocrepis* samples from areas bordering the *H. algirus* range were collected and sequenced for one mitochondrial (*Cytb*) and four nuclear loci (*DNAH3, PRLR, SPTBN1* and *VIM*). Maximum likelihood and Bayesian Inference phylogenetic analyses and a phylogenetic network analysis based on mitochondrial data showed a deep divergence between two *Hemorrhois algirus* lineages, dating from around 2.9 Ma. These lineages correspond to the subspecies *H. algirus algirus* and *H. algirus intermedius* since their geographic distributions are coincident with the ones proposed for them. The deep

mitochondrial divergence between these two subspecies in combination with their reciprocal monophyly at nuclear loci raises the question of whether the *H. algirus* subspecies merit full species status, elevating *H. algirus intermedius* to *Hemorrhois intermedius* Werner, 1929. Given the current data we suggest this nominal change should be not made yet, but requires additional samples and morphological data especially of *H. algirus algirus*.

The phylogenetic network analyses of the four nuclear markers unveiled deep incomplete lineage sorting between *H. algirus* and *H. hippocrepis*, especially at the loci *DNAH3* and *PRLR*. However, the pattern of nuclear haplotype sharing between species observed in individuals from the contact areas indicates also a role of hybridization and introgression in shaping the observed genetic structure. Further studies should include more samples away from the border between the two species' range in order to better characterize pure and hybrid forms and to assess the contribute of incomplete lineage sorting and hybridization in the formation of the phylogeographic pattern observed.

Keywords

Colubridae – *Hemorrhois* – Maghreb – Phylogenetics – Phylogeography

Resumo

A biodiversidade não está homogeneamente distribuída pela Terra. Existem regiões denominadas de "hotspots" (pontos quentes), onde a biodiversidade, em grande parte endémica, está mais concentrada do que em outras. Muitas dessas áreas são pouco estudadas e estão em grande risco devido ao impacto humano. É, por isso, essencial estudá-las para encontrar a melhor maneira de as proteger.

No Norte de África, a região do Magreb faz parte do “hotspot” da bacia do Mediterrâneo. Esta região possui uma grande riqueza em biodiversidade, uma vez que abrange habitats do Mediterrâneo, um deserto extremamente árido, o Sahara, e habitats de montanha, na cadeia montanhosa do Atlas. As dinâmicas do clima e a da distribuição dos habitats ao longo dos últimos milhões de anos moldaram a diversidade genética das espécies encontradas nesta região, como revelaram alguns estudos moleculares recentes. No entanto, a biodiversidade desta região é ainda subestimada devido à instabilidade política e ao difícil acesso a muitas áreas remotas.

A cobra-Argelina, *Hemorrhois algirus*, e a cobra-de-ferradura, *Hemorrhois hipocrepis*, são as duas espécies do seu género a habitar esta região. As duas têm principalmente uma distribuição alopátrica, sendo que a primeira está presente nos habitats semidesérticos do Sul e a segunda nas regiões Mediterrânicas do Norte. Apesar de terem características fenotípicas distintivas, têm sido encontrados muitos indivíduos com características intermediárias em regiões de simpatria, em Marrocos. Os estudos genéticos anteriores destas espécies têm-se focado principalmente nas suas relações filogenéticas. No entanto, *H. hipocrepis* já foi o foco de duas avaliações filogeográficas, uma com fragmentos genéticos mitocondriais e outra com fragmentos nucleares, que descobriram pouca diversidade genética. Por outro lado, não há dados sobre a variação intraespecífica de *H. algirus* e não é clara a natureza das formas morfologicamente intermédias entre as duas espécies. Os principais objetivos deste trabalho foram: (1) avaliar os padrões filogeográficos de *H. algirus*, (2) avaliar a relação filogenética entre as formas puras e intermédias de *H. algirus* e *H. hippocrepis*, (3) e relacionar os resultados com a atual taxonomia intraespecífica da espécie.

Para isso, foram reunidas amostras de *H. algirus*, da maior parte da sua distribuição, e amostras de *H. hipocrepis*, de áreas fronteiriças com *H. algirus*, perfazendo um total de 65, e foram sequenciados um gene mitocondrial (*Citocromo b*) e quatro genes nucleares (*DNAH3, PRLR, SPTBN1*, *VIM*). Análises filogenéticas de máxima verosimilhança (Maximum Likelihood) e inferência bayesiana (Bayesian Inference) e uma análise filogenética de rede (*network*) baseada em dados mitocondriais

mostraram uma divergência profunda entre duas linhagens de *Hemorrhois algirus*, que data de cerca de 2.9 milhões de anos. Essas linhagens correspondem às subespécies *H. algirus algirus* e *H. algirus intermedius*, uma vez que as suas distribuições geográficas são coincidentes as previamente propostas para as mesmas. A divergência mitocondrial profunda entre estas subespécies, combinada com a sua monofilia recíproca nos genes nucleares põe em questão se as subespécies de *H. algirus* não merecerem ser consideradas como diferentes espécies, elevando *H. algirus intermedius* a *Hemorrhois intermedius* Werner, 1929. Com os dados atualmente disponíveis sugerimos que esta mudança nominal não deve ser feita de imediato, pois requer mais amostras e dados morfológicos, especialmente de *H. algirus algirus*.

As análises filogenéticas de rede (*networks*) dos quatro marcadores nucleares revelaram uma separação incompleta de linhagens entre *H. algirus* e *H. hipocrepis*, especialmente nos marcadores *DNAH3* e *PRLR*. No entanto, o padrão de partilha de haplótipos nucleares entre espécies observado em indivíduos das zonas de contacto indicam também um papel de hibridização e introgressão na moldagem da estrutura genética observada. Estudos futuros devem incluir mais amostras longe da fronteira entre estas duas espécies para caracterizar melhor formas puras e híbridas e para inferir o contributo da separação incompleta de linhagens e da hibridização na formação do padrão filogeográfico observado.

Palavras-chave

Colubridae – *Hemorrhois* – Magreb – Filogenética – Filogeografia

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List of Abbreviations

12S	*Mitochondrially Encoded 12S RNA*
95%HPD	95% High Posterior Density interval
aLRT	Shimodaira-Hasegawa-Like support value
BI	Bayesian Inference
Bp	Base pairs
BSA	Boverine Serin Albumine
BV	Bootstrap value
C-mos	*Oocyte maturation factor*
CIBIO	Centro de Investigação em Biodiversidade e Recursos Genéticos
COI	*Cytochrome C Oxidase Subunit I*
Cytb	*Cytochrome b*
DNA	Deoxyribonucleic acid
DNAH3	*Dynein Axonemal Heavy Chain 3*
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
Fig.	Figure
IUCN	International Union for Conservation of Nature
Km	Kilometers
Ma	Million years ago
$MgCl_2$	Magnesium chloride
ML	Maximum Likelihood
mtDNA	Mitochondrial Deoxyribonucleic acid
ND1	*NADH Dehydrogenase Subunit 1*
ND2	*NADH Dehydrogenase Subunit 2*
ND4	*NADH Dehydrogenase Subunit 4*
nuDNA	Nuclear Deoxyribonucleic acid
P.	Page
PCR	Polymerase chain reaction
PP	Posterior probability
PRLR	*Prolactin Receptor*
SDS	Sodium dodecyl sulfate
SPTBN1	*Spectrin Beta, Non-Erythrocytic 1*
VIM	*Vimentin*
Ybp	Years Before Present

Introduction

Biodiversity Hotspots

*«**biodiversity** (…) the number and diversity of distinct living species within the world or a particular environment:* The present reduction of biodiversity … will be the largest setback to life yet – *New Scientist»* (Allen 2007)

No dictionary can really explain the full meaning of biodiversity. Despite that, in its most ancient origins, it could be referent to the diversity of distinct living species, although nowadays this concept encompasses a much broader scale. Biological diversity is now also referred to alternate levels, such as populations, communities and ecosystems, and to smaller levels, like genetic diversity. All these levels of biodiversity are made of dynamic and interactive units that, allied with abiotic factors, are unique features that make part of the diversity of its upper level, and so on. These complex relationships are what makes this area so attractive to scientists, but also what makes it so vulnerable, as one disturbance in one of its units can have repercussions through all of them.

This is why, for years, scientists from around the world have been trying to alert people about the effects of humankind actions, like the recent climate changes (Turner et al. 2010) or massive deforestation and habitat loss (Malhi et al. 2008), which in turn reflect themselves in declines in biodiversity (Pimm et al. 1995; Millenium Ecosystem Assessment 2005; Wake and Vredenburg 2008; Dirzo et al. 2014; Ceballos et al. 2015). All these disturbances in Earth's balance can turn into direct problems for human populations (Ahmed et al. 2009; Ehrlich and Ehrlich 2013) and this is one major reason for us to protect biodiversity from our own mistakes. The worst consequence that can result from human disturbances is species extinction, since it is essentially irreversible. As all living species are a vast genetic storehouse that may contain the cure for present and future diseases waiting to be discovered, any loss could be fatal. In the same way, many of man's inventions and technologies are inspired by nature (Benyus 2009), giving another reason to protect it. Nevertheless, despite any utility they may have to humankind, all living organisms have the same right to live in this planet, which should be the main reason for species conservation. However, to protect biodiversity, we need to know it and therefore, we need to study it. In fact, the International Union for Conservation of Nature (IUCN) has only evaluated the threat status of around 59% of vertebrate species, within which Reptiles, Amphibians and Fishes have only 44%, 88%

and 38% of species evaluated, respectively (Ceballos et al. 2015). All this shows there is still a long way for us to have all the information we need to know with certainty how and where to act to prevent most of the losses that are in our path.

Biodiversity in terms of species diversity is not homogeneously distributed on Earth (Myers et al. 2000; Mittermeier et al. 2004; Mittermeier et al. 2011). There are regions called “hotspots” where biodiversity, and especially endemic diversity, is more highly concentrated than others (Myers et al. 2000; Mittermeier et al. 2004; Mittermeier et al. 2011; Fig.1). Many of these areas are also at great risk because of human impact (Mittermeier et al. 2011). For a region to be considered a hotspot it needs to have at least 1500 vascular plants as endemics (>0.5% of the world’s total) and it needs to have 30% or less of its original vegetation (Myers et al. 2000; Mittermeier et al. 2004; Mittermeier et al. 2011). These hotspots contain 50% of the vascular plants and 43% of the terrestrial vertebrates (Mittermeier et al. 2004; Mittermeier et al. 2011) in just 2.3% of the world’s land area, though their original extent was 15.9% (Mittermeier et al. 2011). That is why these hotspots play a critical role for conservation, since human disturbance in these areas may lead to huge biodiversity losses.

However, there are regions in the world that may not be considered hotspots because they are still poorly studied, what has made it necessary to update the number of hotspots as more information was made available (Mittermeier et al. 2004; Mittermeier et al. 2011). The regions with the best economic resources and facilities are the ones that have their biodiversity better described, due to their level of attractiveness for researchers. Furthermore, regions with harsh conditions, like hot deserts, tend to be less

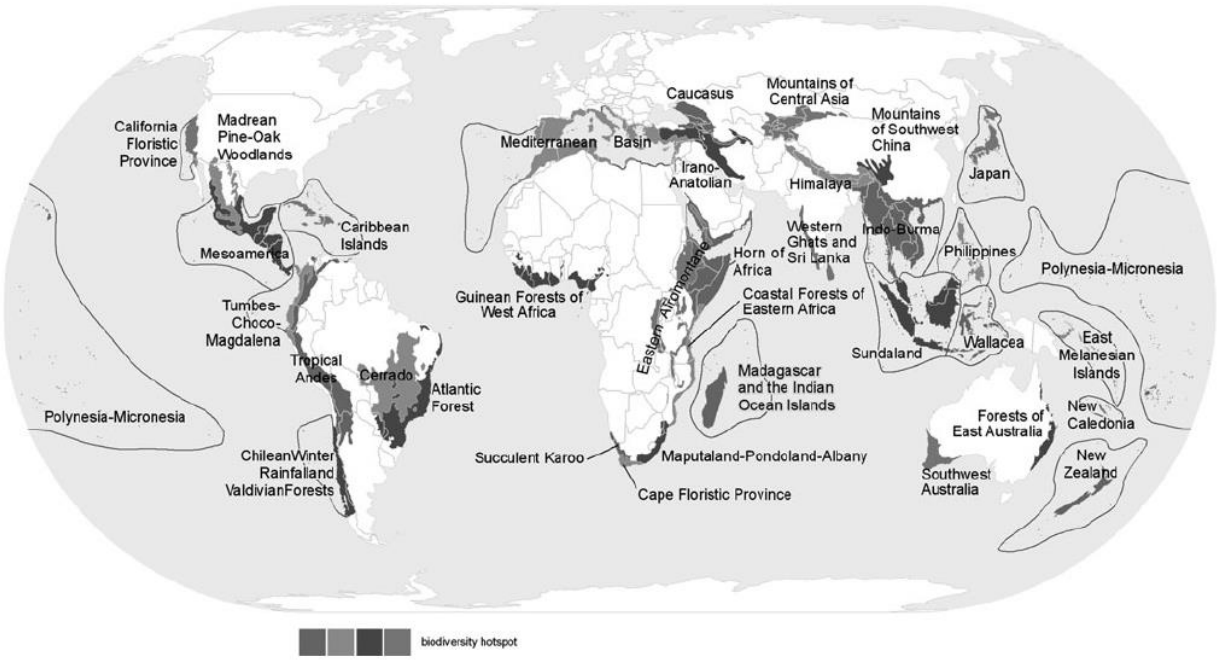

Figure 1 – The world’s biodiversity hotspots. (Taken from *Mittermeier et al. 2011*)

explored, not only when combined with not so good economic resources, but also because they are perceived to have rather homogeneous areas and low diversity (Durant et al. 2012). In fact, this is not totally true because deserts and arid regions tend to have species distributed in patches whose range is affected by their climatic limitations and a relatively high rate of endemism due to those organisms' adaptations to the desert extreme environment, which creates some located micro-hotspots of biodiversity as, for instance, in mountain regions (Davies et al. 2012; Murphy et al. 2012; Ficetola et al. 2013).

In these regions still poorly explored in biological terms, despite the recent growing number of studies (e.g. in the Sahara/Sahel; reviewed in Brito et al. 2014), knowledge regarding the diversity and distribution of many organisms, such as reptiles, and particularly snakes, tend to still be incomplete. In addition to the difficulty of carrying out biological surveys in these regions, there are also two notable taxonomic problems. One is the occurrence of cryptic species (i.e. two or more species which are genetically distinct but morphologically indistinguishable), which are assigned to a single nominal species determining a biodiversity underestimation (Bickford et al. 2006), since there is no information about the variability of many organisms. The second one is the taxonomic assignment of morphologically intermediate forms between sister taxa, since the number of known specimens is low such as in the case of snakes and it is difficult to determine if these are rare morphological abnormalities, if they represent hybrids or if they actually correspond to distinct genetic entities (e.g. Marín and Barroso 2012). These two problems can be effectively overcome by combining molecular phylogeography and phylogenetics with morphological data, which can provide an accurate assessment of species delimitation and relationships as well as of geographic pattern of biodiversity distribution across the study region (e.g. Perera and Harris 2010; Barata et al. 2012; Sampaio et al. 2015; Rosado et al. 2017; Salvi et al. 2017a).

The Maghreb

The Maghreb region, in North Africa, is home to a great amount of biodiversity and includes part of the Mediterranean basin, one of the world's biodiversity hotspots (Fig. 1; Myers et al. 2000; Mittermeier et al. 2004; Cuttelod et al. 2008; Mittermeier et al. 2011). However, it is still a region poorly studied and with considerable biodiversity waiting to be described (Ficetola et al. 2013). The Maghreb region includes regions of Libya, Tunisia, Algeria, Morocco, the Western Sahara and Mauritania. These countries are former French, Italian and Spanish colonies and most of them suffered from

oppressive regimes, civil and regional wars and terrorist attacks that made it difficult to conduct scientific research in the region after their independence in the 1950s, 60s and 70s.

This region is characterized mainly by Mediterranean climate and habitats, the largest arid desert in the world, the Sahara desert, and high mountain areas such as the Atlas Mountains (Le Houerou 1997), which creates lots of unique habitats that host many unique species (Sochurek 1979; Bons and Geniez 1996; Geniez et al. 2000; Fahd and Pleguezuelos 2001; Habel et al. 2012; Jaskuła 2015).

North Africa did not always have the climatic conditions that enable these habitats.

Overall, the Cenozoic has been marked globally by a general succession that transformed the Paleogene (66-23 million years ago, Ma) warm-humid greenhouse climate into the Quaternary (2.6 Ma to present) glacial and interglacial period (Retallack 2001; Zachos et al. 2001). During the Paleogene, Africa was dominated by tropical forests (Jacobs et al. 2010), with some grasslands appearing in North Africa by the Eocene (Jacobs 2004; Jacobs et al. 2010). In the Early and Middle Miocene (23-11.6 Ma), North Africa still had a warmer and more humid climate, dominated by tropical trees (Jacobs 2004; Jacobs et al. 2010), but towards its end the vegetation turned drier with the appearance of open grasslands and scrublands (Jacobs 2004; Jacobs et al. 2010; Pound et al. 2012). However, in the Tortonian (11.6-7.2 Ma) there were still sufficient humid conditions to enable North African rivers heading to the Mediterranean sea (Köhler et al. 2010). The increasing aridity towards the end of the Miocene led to the first appearance of the Saharan desert at around 7 Ma (Le Houerou 1997; Pound et al. 2012).

In the Pliocene (5.3-2.6 Ma), Africa was under a monsoonal climate until around 2.8 Ma, when it started to alternate between wet/hot and dry/cold intervals in response to the glaciations (DeMenocal 1995; DeMenocal 2004; Jacobs et al. 2010). The beginning of the glacial cycles developed gradually between 3.1 and 2.6 Ma, around the Pliocene-Pleistocene border, in line with an African aridification (DeMenocal 1995). Starting with smaller cycles until 1 Ma, when glacial climate extremes markedly increased (DeMenocal 1995), the African continent responded with enhanced aridity and cooler temperatures and the expansion of the Sahara during glacial maxima and with more humid and hotter conditions during the interglacial periods (DeMenocal 1995; DeMenocal 2004; Jacobs et al. 2010).

The Holocene (11500 ybp to present) marked North Africa with the transition from a humid period where a “green Sahara” appeared (Claussen and Gayler 1997; Claussen et al. 2003) to today’s largest warm desert of the world. This “green Sahara” was covered by grasses (Poaceae) with capacity to host some xerophytic woods and even warm

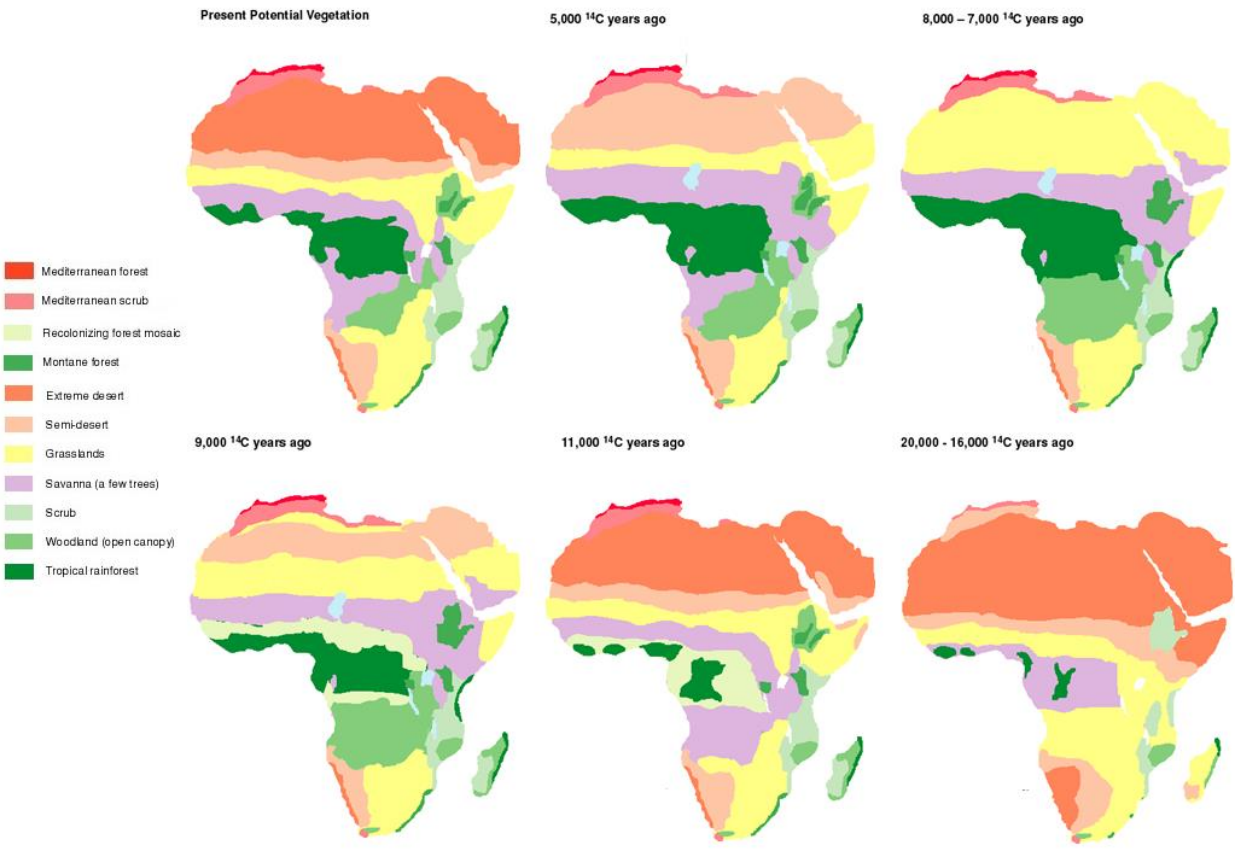

Figure 2 – The evolution of vegetation land cover in Africa through the last 20000 years. Adapted from http://www.esd.ornl.gov/projects/gen/nercAFRICA.html

mixed forest (Prentice et al. 2000; Claussen et al. 2003; Kröpelin et al. 2008), though it still had some desert areas (Brovkin et al. 2002). Before 4300 ybp it started to become more arid, being covered by open grass savannah (Kröpelin et al. 2008). At around 3700 ybp, the modern regional wind regime established itself, leaving, by 2700 ybp the vegetation found today throughout the central Sahara (Kröpelin et al. 2008).

With all these climatic changes throughout the last million years, species always needed to adapt, migrate, accordingly to their dispersal capabilities, or face local extinction. During glacial periods, lots of water was trapped in the ice sheets, turning the atmosphere drier in lower latitudes, with the opposite happening in interglacial periods. In Europe, temperate animals and plants shifted southwards to find refugia in the European peninsulas during each glacial phase (Hewitt 1996; Hewitt 2000). In North Africa, the appearance of an enormous desert promoted by the drier atmosphere forced animals and plants to find refugia north, in the Maghreb, south, replacing rain forests, or upwards, taking refuge in the North African mountains (Arctander et al. 1999; Hewitt 2000; Hewitt 2001; Hewitt 2004; Kröpelin et al. 2008). Despite this, the appearance of the Saharan desert also let species adapted to aridity to thrive and expand their ranges (Rato and Harris 2008; Gonçalves et al. 2012; Metallinou et al. 2015). On the other hand, in the interglacial periods, North African arid life forms experienced a contraction of their

ranges and the ones sheltered in more humid environments would expand theirs to the “greener” Sahara (Cosson et al. 2005). These cycles of expansion and contraction meant that populations experiencing isolation in refugia had gone through allopatric differentiation and further in time they experienced secondary contact (Cosson et al. 2005), shaping the genetic diversity observed within the species today.

The Maghreb is also crossed by a series of West-East oriented mountain chains: the High Atlas, the Middle Atlas, the Saharan Atlas, the Tell Atlas and the Aurès Mountains, which together form the Atlas mountain belt, and the Anti-Atlas. The Atlas Mountains are located in the North-western part of the African plate and are a result of the collision of this plate with the Eurasian plate (de Lamotte et al. 2009; Babault et al. 2012). The elevation of the Atlas started out with a first uprising event by the Middle Eocene and Oligocene, around 50-23 Ma (Bracène and de Lamotte 2002; de Lamotte et al. 2008; Robert-Charrue and Burkhard 2008; de Lamotte et al. 2009). Furthermore, a second and bigger event started in the Late Miocene-Pliocene and it continues until today (Babault et al. 2008; de Lamotte et al. 2008; Robert-Charrue and Burkhard 2008; de Lamotte et al. 2009).

Although all the system has been rising together in these periods of time, there is a consistent asymmetry in it, with the west part being much higher than the east (de Lamotte et al. 2009). In fact, the High Atlas present a mean elevation of 1500 m, contrasting with the mean 1050 m and 600 m in the Saharan Atlas and in the Aurès/Tell Atlas, respectively (de Lamotte et al. 2009). This asymmetry is caused by the fact that, in the western Atlas, the continental crust was only slightly thinned during pre-orogenic times (Missenard et al. 2006; Babault et al. 2008; de Lamotte et al. 2008). As for the Anti-Atlas, although it is only separated from the High Atlas by narrow foreland basins, it has a different origin than the Atlas system, nevertheless its formation was contemporary and very influenced by its northern bigger neighbour (Michard et al. 2008; Zouhri et al. 2008; de Lamotte et al. 2009).

These North African mountains are surrounded by peripheral plateaus and plains that interconnect them (Babault et al. 2008; de Lamotte et al. 2009), which are crossed by both permanent and seasonal rivers with origin in the mountain peaks that hydrate the lower altitude soils. However, these rivers are mainly directed to the Mediterranean and Atlantic slopes (de Lamotte et al. 2008; Babault et al. 2012), which leads to a huge contrast when we compare northwest cedar forest slopes with southeast arid slopes, in Morocco (de Lamotte et al. 2008). Both the presence of high mountains, that permit the succession of ecosystems along their altitude, and the preferential drainage of the river waters have very important contributions to the diversity of this region.

The role of Phylogeography

All the climatic and orogenic processes mentioned above have considerable impacts in the populations that inhabited and still inhabit the Maghreb. These processes can join and separate populations, they can modify habitats' distribution and they can give rise to species fragmentation (and ultimately speciation) or extinction. These populations dynamics can be studied to understand the role these processes had in shaping the present genetic distributions of species. For this purpose, phylogenetic and phylogeographic methods are the most important tools.

Phylogeography is a discipline that was outlined in 1987 (Avise et al. 1987), with the objective of conciliating two different disciplines: phylogenetics (that deals with macroevolution) and population genetics (that deals with microevolution). The name of this new discipline informs that it deals with the geographic distribution of genetic lineages, particularly within and among closely related species, but also aims to comprehend the historical processes of evolution that led to these lineages distribution in the first place (Avise 1998; Avise 2000; Avise 2009; Hickerson et al. 2010). Hence, phylogeography enables the study of processes of speciation through lineage distribution, to identify lineage contact zones and hybrid zones (Avise et al. 1987; Avise 2000).

The studies of phylogeography began with the use of mitochondrial DNA (mtDNA) to uncover patterns of spatial arrangements of lineages within species. The characteristics of mtDNA make it an ideal marker for easy and quick phylogeographic analyses. First, it has a high mutational rate, compared with nuclear DNA (nuDNA), which implies high intraspecific variation and easier establishment of lineages (Brown et al. 1979; Ballard and Whitlock 2004). Another very important feature of mtDNA is the fact that each individual animal typically carries only one non-recombining mitochondrial genotype with maternal inheritance that exists in multiple copies in each cell, making it easier to extract and amplify. However, its exclusive maternal transmission in most animals also means that mtDNA can only inform about matrilineal histories of populations which may or may not be similar to the actual history of the whole population (Avise 2000; Ballard and Whitlock 2004; Avise 2009).

To overcome this problem, scientists started using also nuDNA, which effectively has a maternal and paternal inheritance, but now they faced other problems. Unlike mitochondrial loci, nuclear loci typically have a slow pace of sequence evolution, they appear in pairs, in diploid organisms, that may or may not be identical (i.e. be homo- or heterozygotic) and go by a process of recombination during the formation of gametes,

which can mix strings of DNA with different evolutionary histories (Avise 2000; Avise 2009). To surpass the first problem, scientists identified nuclear sequences that evolve faster than average and for the second one, various programs were developed to sort out haplotypes in heterozygotic sequences (Garrick et al. 2010). As for the recombination hurdle, the use of sequences from short gene fragments minimizes the probability of recombination; still available tools allow testing for recombination and recombinant data can simply be discarded. With these advances, nuDNA has been successfully employed in many studies, and now it is considered essential for phylogeographic assessments (e.g. Godinho et al. 2008; Fonseca et al. 2009; Salvi et al. 2017a).

Phylogeographic studies deal with various populations distributed across the species range. There are always impediments to dispersal related to species limitations, like their dispersal ability, behaviour, feeding needs and physical or environmental barriers such as for example mountains or patches of unsuitable habitat. Such barriers to dispersal can change through time. Dramatic contraction and expansion of habitats have taken place during Pleistocene climatic changes (DeMenocal 1995; DeMenocal 2004; Jacobs et al. 2010), determining isolation and divergence between populations and secondary contact with genetic admixture between divergent lineages (Hewitt 2000). The main goal of phylogeographic studies is to use gene trees to infer the past and present forces that have produced the current genetic structure of populations and closely related species. Therefore, the assessment of gene trees from multiple unlinked loci (nuclear and mitochondrial) is important in order to identify patterns of inter-locus genealogical concordance (or lack of them) that will give better interpretations for the historical processes underlying current geographic patterns of genetic diversity (Avise 2000; Avise 2009).

Study species

Two of the four whip snakes species of the genus *Hemorrhois* Boie, 1826 occur in the Maghreb: *Hemorrhois hippocrepis* Linnaeus, 1758, and *Hemorrhois algirus* Jan, 1863 (Bons and Geniez 1996). The other two species, *Hemorrhois nummifer* Reuss, 1834, and *Hemorrhois ravergieri* Ménétries, 1832, occur in Central Asian and the Middle East. These species of the family Colubridae Oppel, 1811, were previously assigned to the genus *Coluber* Linnaeus, 1758, before the genus *Hemorrhois* was recognized, in 2001 (Schätti and Utiger 2001). Inside the Colubridae family, this genus seems closely related to the genera *Platyceps* and *Spalerosophis* (Nagy et al. 2004; Pyron et al. 2011; Pyron et al. 2013), which include snakes from Africa and Asia.

Hemorrhois hippocrepis, the Horseshoe whip snake, is a medium-sized snake that normally does not pass 120 cm long, though exemplars with more than 150 cm have been reported (Pleguezuelos and Feriche 1999; Carranza et al. 2006; Vigara 2012; Feriche 2015). This snake has a characteristic “horseshoe” mark in the neck and a pattern of dark rounded marks along its body, with a very variable basic colour that is usually greyish, yellowish, reddish or brownish (Bons 1962; Schleich et al. 1996; Vigara 2012; Feriche 2015). In spite of the fact that its typical pholidosis is characterized by the existence of a row of sub-ocular scales separating the supralabial scales from the eye (Bons 1962; Schätti 1986; Schleich et al. 1996; Marín and Barroso 2012; Feriche 2015), there have been found individuals with one or two supralabials in contact with the eye (Bons 1962; Schätti 1986; Marín and Barroso 2012; Feriche 2015). When this occurs, it always involves the 5th and/or the 6th supralabial and it can also be present on only one side of the snake’s head or with a different pattern on each side (Bons 1962). The dorsal scales reach 25-29 rows at mid-body, very rarely 23 (Bons 1962; Schätti 1986; Schleich et al. 1996; Feriche 2015).

It inhabits the Iberian Peninsula, the Maghreb region and the Mediterranean islands of Pantelleria and Sardinia (Fig. 3; Carranza et al. 2006; Feriche 2015), though

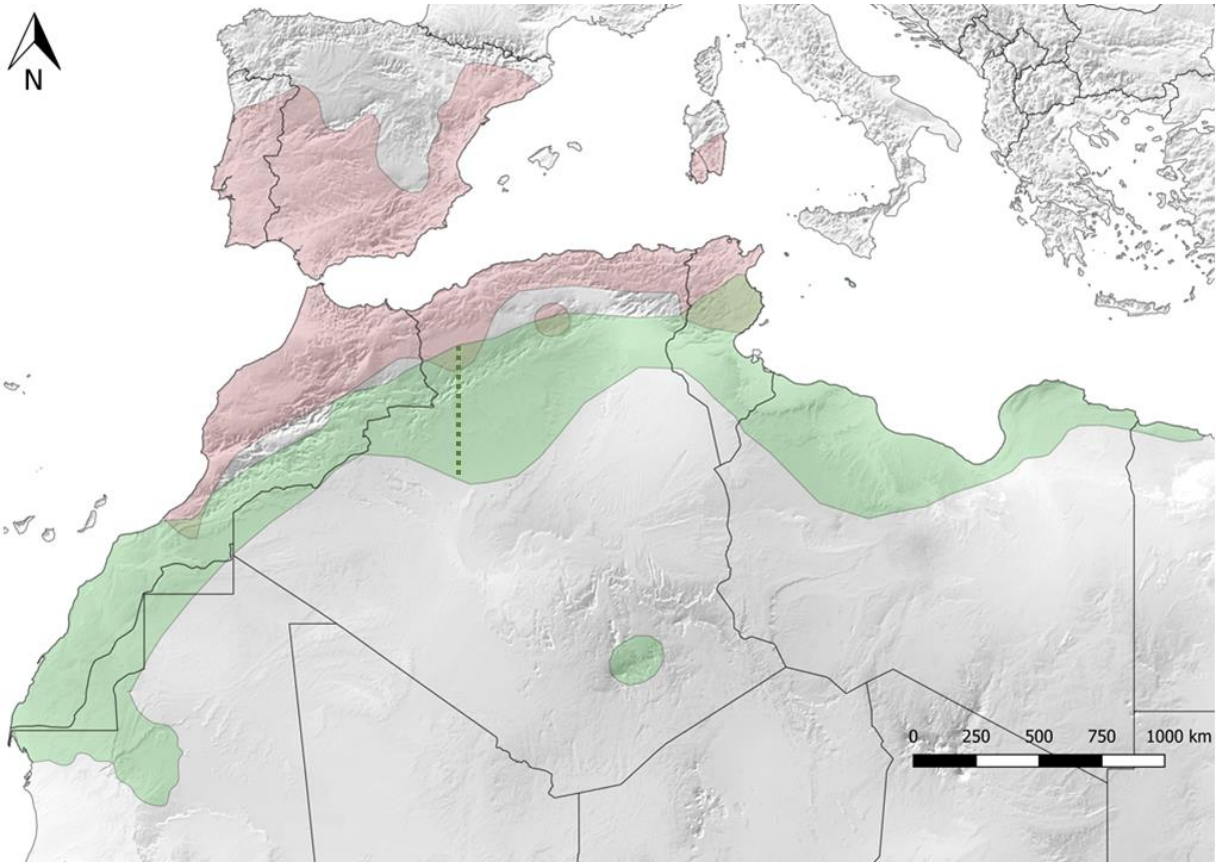

Figure 3 – Distribution map of *Hemorrhois hippocrepis* (in pale pink) and *Hemorrhois algirus* (in pale green). The spatial data was downloaded from each species’ IUCN Red List webpage (Miras et al. 2009; Wagner and Wilms 2013). The green dotted line represents the separation between *H. algirus algirus*, distributed to the east, and *H. algirus intermedius*, distributed to the west, according to Schätti (1986).

it was recently introduced in the Balearic islands (Feriche 2015; Silva-Rocha et al. 2015). The population of Pantelleria is considered a different subspecies: *Hemorrhois hippocrepis nigrescens* Cattaneo, 1985; while in the remaining distribution all exemplars belong to the nominal subspecies, *Hemorrhois hippocrepis hippocrepis* (Carranza et al. 2006). In North Africa, it is distributed through the areas of Mediterranean climate (Bons and Geniez 1996) and it is very common and found in every type of habitat, including in human environments (Fahd and Pleguezuelos 2001; Feriche 2015).

Hemorrhois algirus, the Algerian whip snake, is the sister-species of *H. hippocrepis* (Nagy et al. 2004; Wüster et al. 2007; Pyron et al. 2011; Pyron et al. 2013), though it is a bit smaller than *H. hippocrepis*, reaching a maximum length of 140 cm, but usually not surpassing 100 cm (Sochurek 1979; Schätti 1986). Rarely there are found individuals that can have the “horseshoe” mark characteristic of its sister-species, but there are also individuals with a uniform grey or black head (Schleich et al. 1996; Geniez et al. 2004; Marín and Barroso 2012). It has a greyish, orange, green or brownish base coloured body with darker designs of transversal bars or also rounded patterns along it, like *H. hippocrepis* (Schleich et al. 1996; Marín and Barroso 2012). Furthermore, its pholidosis is characterized for having 1 or 2 supra-labials in contact with the eye, always the 5th and/or the 6th (Bons 1962; Schleich et al. 1996; Marín and Barroso 2012). The

Figure 4 – Exemplars of *Hemorrhois hippocrepis* (top left), *H. algirus intermedius* (bottom left) and *H. algirus algirus* (right). Location and credits from top to bottom and left to right: Casablanca, Morocco, photo by Raúl León; Guelmim, Morocco, photo by Philippe Geniez; Tunisia, photo by Tomas Mazuch; Tunisia, photo by Tomas Mazuch.

dorsal scales are disposed at 23-25 rows at mid-body (Bons 1962; Schätti 1986; Schleich et al. 1996), or 27 rows in the isolated population of Malta (Bons 1962).

This semi-arid adapted snake occurs in a north Saharan string covering Egypt, Libya, Tunisia, Algeria, Morocco, the Western Sahara and Mauritania, and also on the islands of Malta (Fig. 3; Bons 1962; Bons and Geniez 1996; Geniez et al. 2000; Padial 2006). It is represented by two subspecies: *Hemorrhois algirus algirus*, the nominal one, that is present in its eastern range, from West Algeria to Egypt (Bons 1962; Schätti 1986); and *H. algirus intermedius* Werner, 1929, that is present in the western part of the range, from West Algeria to Mauritania (Schätti 1986; Bons and Geniez 1996). A third subspecies was described in the south of Morocco, *Hemorrhois* (*Coluber*) *algirus villiersi* Bons, 1962, but it was posteriorly considered synonym to *H. algirus intermedius* (Schätti 1986). This snake appears to have an unclear distribution in zones near the territory of *H. hippocrepis* (Marín and Barroso 2012). Additionally, it is very difficult to identify to the species level some individuals from these "contact" zones with *H. hippocrepis* due to apparent intermediate morphologies (Bons 1962; Bons and Geniez 1996; Marín and Barroso 2012), especially in South Morocco, in the south-eastern High Atlas and Anti-atlas slopes.

In fact, the peculiar climate and environments present in the south of Morocco, that seems to be a transition zone between coastal, mountain and arid climates (Brito et al. 2011) allows snakes with different affinities to coexist in sympatry (Brito et al. 2011; Marín and Barroso 2012). In the case of whip snakes, in such area of sympatry it is difficult to differentiate them from each other, with specimens found to have various combinations of rounded patterns or transversal bars with "horseshoe" marks or grey or black heads, or even unusual very pale typical *H. hippocrepis* patterns (Marín and Barroso 2012). These individuals from contact zones that are difficult to attribute to one species are usually assigned to *H. a. intermedius*, though even the taxonomic validity of this subspecies has been questioned (Schleich et al. 1996).

Previous studies

A study performed by Schätti and Utiger (2001) used a total of three *H. hippocrepis* samples from Morocco and one *H. algirus* sample from Tunisia and they presented two phylogenetic trees based on mitochondrial *COI* and *12S* gene fragments. From these trees, they concluded that molecular data supported earlier findings based on morphological evidence, which proposed that *Coluber nummifer* and *C. ravergieri*

were closely related to *H. algirus* and *H. hippocrepis*, therefore the genera *Hemorrhois* should be formally used for these four species.

Similar results were obtained by Nagy et al. (2004) with one *H. hippocrepis* sample from Spain and one *H. algirus* sample from Tan-Tan plage (Morocco), near *H. hippocrepis* territory. The phylogenetic trees presented, based on *Cytb*, *ND1*, *ND2* and *ND4* mitochondrial gene fragments, show a strong support of the *Hemorrhois* clade. However, the authors also indicated that the *C-mos* nuclear gene analysis did not support the *H. algirus* and *H. hippocrepis* clade.

In a phylogenetic study concerning the African spitting cobras, Wüster et al. (2007) used the *Cytb* and *ND4* mitochondrial gene fragments from the four *Hemorrhois* species sequenced and shared in GenBank by Nagy et al. (2004) to calibrate a tree. They proposed a maximum of 16 Ma for the divergence between the African and Asian clades of this genus, since this was approximately the time since Asia and Africa became joined allowing the dispersal of *Hemorrhois* in Africa and the onset of the divergence with Asian lineages.

The sequences of mitochondrial *ND2*, *ND4* and *Cytb* gene fragments and nuclear *C-mos* gene fragment shared in GenBank by Nagy et al. (2004) and Carranza et al. (2006) were also used by Pyron and colleagues for building mega-phylogenies of the sub-family Colubroidea (Pyron et al. 2011) and of Squamata (Pyron et al. 2013). They obtained the same results of the previous phylogenetic studies with a strong support for the monophyly of *Hemorrhois* (Shimodaira-Hasegawa-Like support value, aLRT: 100%) and for the sister relationships between *H. algirus* and *H. hippocrepis* (aLRT: 100%) and between *H. nummifer* and *H. ravergieri* (aLRT: 100%).

The first intraspecific study on genetic variation was carried out by Carranza et al. (2006) on *H. hippocrepis*. These authors analysed mitochondrial *12S* and *Cytb* gene fragments from 37 individuals mainly from Spain and North Morocco, and found low differentiation between populations from the two sides of the strait of Gibraltar (Fig. 5). A sample from one *H. algirus* individual from Tunisia was also used for a phylogenetic analysis. The authors concluded that *H. hippocrepis* had a North African origin, where it has also its sister species, *H. algirus*, and that the two diverged around 4-7 Ma.

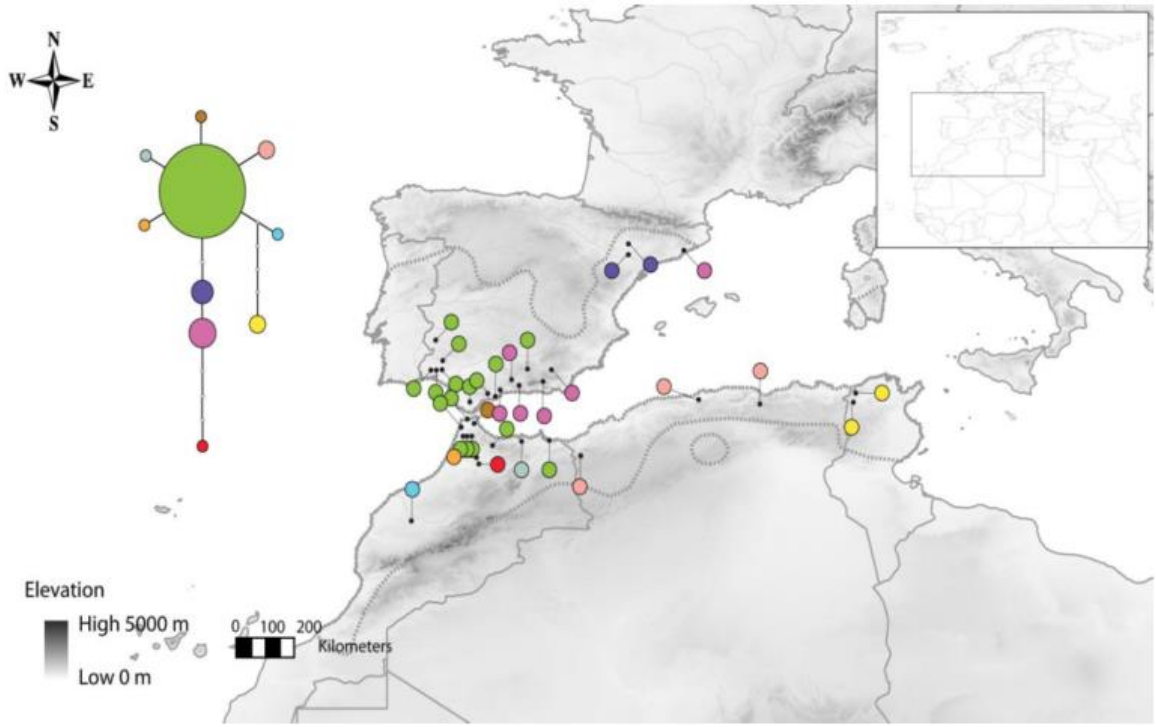

Figure 5 – Results from the *Hemorrhois hippocrepis* phylogeographic study performed by Carranza et al. (2006). It represents the samples distribution and haplotype network inferred from 300bp of *Cytb* and 395bp of *12S*. The white circles represent a mutational step. The range of the species is represented by the dashed line. This is an adapted figure taken from Machado (2012).

Finally, in his MSc thesis, Machado (2012) used *BDNF* and *MC1R* nuclear gene fragments to compare nuclear marker patterns of *H. hippocrepis* with the mitochondrial ones inferred by Carranza et al. (2006). Using a total of 38 samples mostly from the Iberian Peninsula and Morocco, the author found again low genetic diversity in the species and reinforced the theory of a recent migration of the species from the Maghreb to the Iberian Peninsula (Figures 6 and 7).

So, in conclusion, many molecular studies including both *Hemorrhois algirus* and *H. hippocrepis* had either a phylogenetic focus (Schätti and Utiger 2001; Nagy et al. 2004; Wüster et al. 2007; Pyron et al. 2011; Pyron et al. 2013) or a phylogeographic focus on *H. hippocrepis* (Carranza et al. 2006; Machado 2012) so they only analysed one individual of *Hemorrhois algirus*. Thus, an assessment of the intraspecific variation of *Hemorrhois algirus* is still lacking.

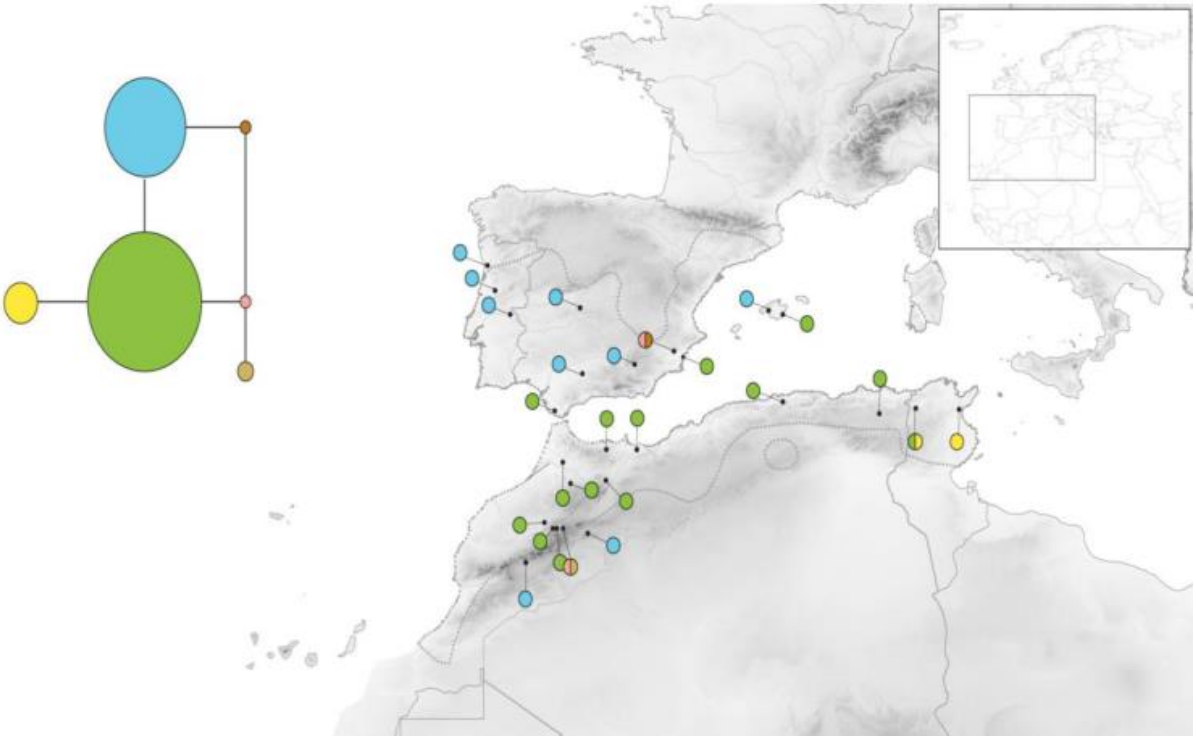

Figure 6 – Results of Machado (2012) for the *BDNF* nuclear gene fragment. It represents a statistical parsimony network based on *BDNF* haplotypes observed in 30 samples of *Hemorrhois hippocrepis* and the geographical distribution these haplotypes. The connections between haplotypes represent a single mutational step. Image taken from Machado (2012).

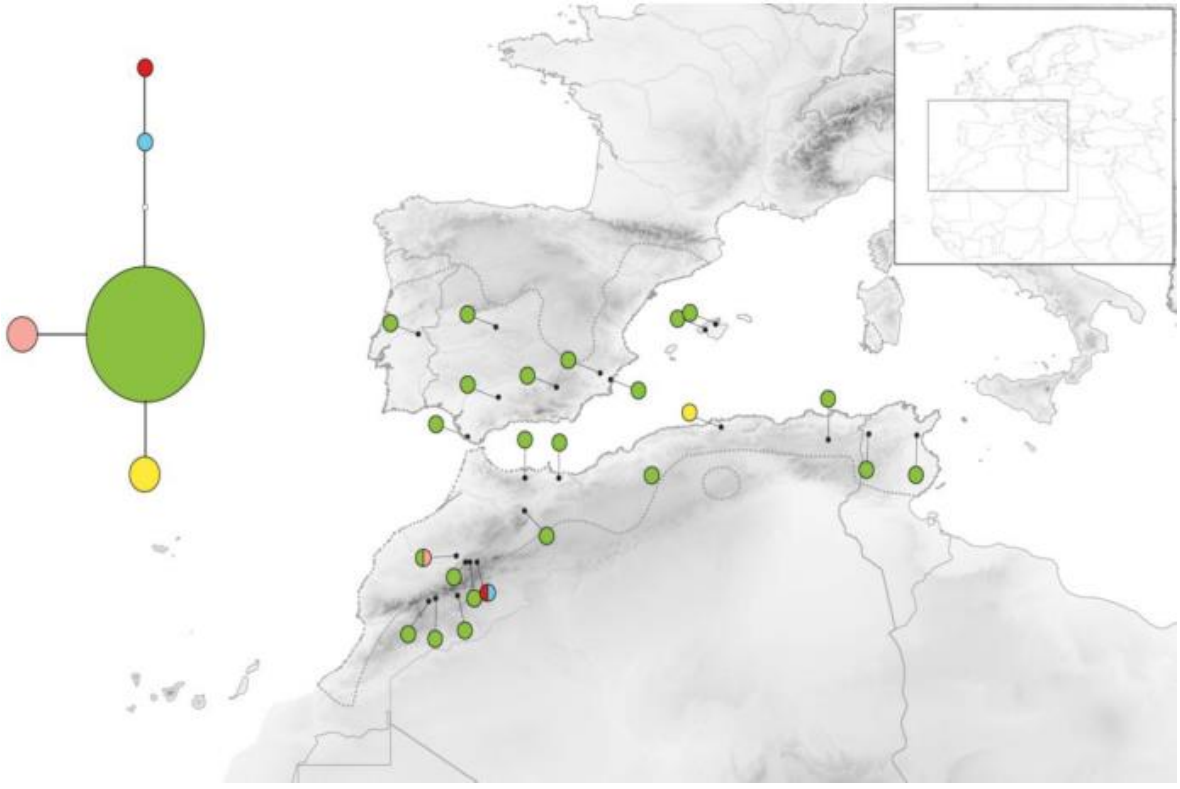

Figure 7 - Results of Machado (2012) for the *MC1R* nuclear gene fragment. It represents a statistical parsimony network based on *MC1R* haplotypes observed in 30 samples of *Hemorrhois hippocrepis* and the geographical distribution these haplotypes. The connections between haplotypes represent a single mutational step. Image taken from Machado (2012).

Aim of the Study

Despite the availability of a phylogeographic assessment of *H. hippocrepis*, none of the previous studies has ever included more than one *H. algirus* sample. This way, we still don't have data on the intraspecific variation of *H. algirus* and is not clear whether intermediate forms between *H. hippocrepis* or *H. algirus* represent morphotypes of one of either species or hybrids between them or even a third undescribed species. The main objectives of this work are:

1. To assess the phylogeographic pattern of variation of *H. algirus*, using both mitochondrial and nuclear data;
2. To assess the phylogenetic relationship between the pure and intermediate forms of *H. algirus* and *H. hippocrepis*, with a dense sampling in the area of range overlap between the two species;
3. To relate the results obtained for the previous objectives with the current intraspecific taxonomy of the species.

Materials and Methods

Sampling

A fieldwork session to Morocco was carried out from the 29th of August to the 9th of September 2016, by my coordinators and I, and other members of the Applied Phylogenetics group from Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO) of the Faculty of Sciences of the University of Porto. Additionally, I used samples from the Applied Phylogenetics group data base, which were previously collected by researchers. Furthermore, these samples analysed were complemented by samples from the collections of Dr José Carlos Brito, Dr Fernando Martinez-Freiría, Dr Philippe Geniez and Dr Gabriel Marín.

The final dataset was composed by all the samples of *Hemorrhois algirus* available, in order to perform a range-wide phylogeographic assessment of this species, and all the available samples of *Hemorrhois hippocrepis* available that had been captured near its range's border with *Hemorrhois algirus* (Fig. 8), in a total of 65 samples

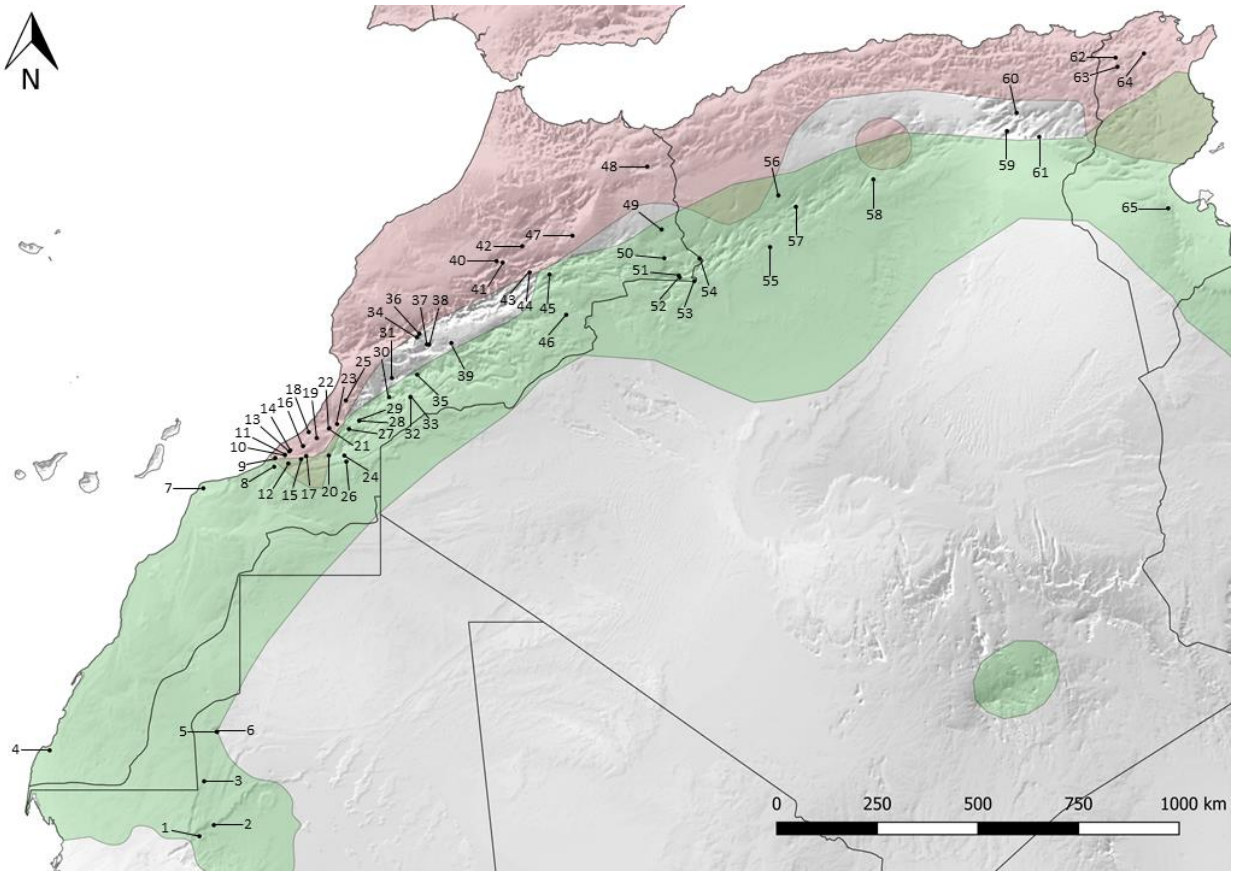

Figure 8 – Map with the location of all the *Hemorrhois algirus* and *Hemorrhois hippocrepis* samples analysed. The distributions of *H. algirus* and *H. hippocrepis* are represented in pale green and pale pink, respectively (Miras et al. 2009; Wagner and Wilms 2013). For more information about the samples see Table 2.

(Table 2). The *H. hippocrepis* samples included some of the ones analysed by Machado (2012). The *H. algirus* dataset included samples from Mauritania, the Western Sahara, Morocco, Algeria and Tunisia, being only lacking samples of *H. algirus* from Libya and Egypt. Also, one sample of *H. nummifer* and four of *H. ravergieri* were included.

DNA extraction, amplification and sequencing

Total genomic DNA extraction from muscle tissue preserved in 99% ethanol was performed following a standard high-salt protocol (Sambrook et al. 1989). The method starts with the addition of 600 µL of lysis buffer (0.5M tris, 0.1M EDTA, 2% SDS, pH 8.0) and 10-12 µL of proteinase K to small amount of tissue (previously cut into pieces of about 25 mg) and placed into 2 mL eppendorf tubes. In this step, tissue lysis is inducted and is maximized by incubating at 56ºC, overnight and with agitation. After the tissue is digested, the eppendorf tubes are placed in the freezer for 10 min. Then, 300 µL of ammonium acetate are added, followed by centrifugation at 14000 rotations per minute (rpm) for 15 minutes, at -4ºC. The resulting supernatant is transferred to a new 2 mL eppendorf tube, to which 600 µL of ice-cold isopropanol are added, followed by a short manual agitation. The samples are then put overnight in the freezer. In the following step, tubes are centrifuged at 14000 rpm for 30 min at -4ºC and the resulting supernatant is discarded. Then, 1000 µL of ice-cold ethanol are added to the pellet and the eppendorf tubes are centrifuged a last time at 14000 rpm for 15 minutes at -4ºC. The supernatant is discarded again and the tubes are incubated at room temperature for a few hours to allow all the ethanol to evaporate. Finally, 100 µL of ultra-pure water are added and the eppendorf tubes are left hydrating at room temperature, overnight.

From this genomic DNA extraction, 5 gene regions were amplified, following a polymerase chain reaction procedure (PCR): the mitochondrial *Cytochrome b* (*Cytb*) gene fragment, two nuclear coding gene fragments, *Dynein Axonemal Heavy Chain 3* (*DNAH3*) and *Prolactin Receptor* (*PRLR*), and two non-coding nuclear genes: *Spectrin Beta, Non-Erythrocytic 1* (*SPTBN1*) and *Vimentin* (*VIM*). The primers used and their respective references are listed in Table 1. The *Cytb* gene was chosen because it was already effectively used for assessing *H. hippocrepis* diversity (Carranza et al. 2006). As this study showed a relatively low structure for the fragment, I chose to change one of the primers to increase its length and to maximize the probability of finding variation and enhance the detection of population structure. The *DNAH3* and *PRLR* fragments appeared to have evolutionary rates substantially higher than other commonly used loci in squamate phylogenetics (Townsend et al. 2008). As for the *SPTBN1* and *VIM*

fragments, they both showed strongly supported results when combined with mitochondrial genes in analyses with Colubrids (Pyron and Burbrink 2009; Chen et al. 2014). Also, *SPTBN1* was recently confirmed to have more variability, in snakes of the genus *Zamenis*, than *C-mos* (*oocyte maturation factor*), a gene widely used in squamates (Salvi et al. 2017b).

Gene	Primer name	Sequence (5'-3')	Source
Cytb	Cytb1	CCA TCC AAC ATC TCA GCA TGA TGA AA	(Kocher et al. 1989)
	CytbR	AAA TAG GAA GTA TCA CTC TGG TTT	(Moritz et al. 1992)
DNAH3	DNAH3_f1	GGT AAA ATG ATA GAA GAY TAC TG	(Townsend et al. 2008)
	DNAH3_r6	CTK GAG TTR GAH ACA ATK ATG CCA T	
PRLR	PRLR_f1	GAC ARY GAR GAC CAG CAA CTR ATG CC	(Townsend et al. 2008)
	PRLR_r3	GAC YTT GTG RAC TTC YAC RTA ATC CAT	
SPTBN1	SPTBN1-F	TTG GTC GAT GCC AGT TGT A	(Chen et al. 2014)
	STPBN1-R	CAG GGT TTG TAA CCT KTC CA	
VIM	VIM_Ex5_F2	AAC AAT GAT GCC CTG CGC CA	(Pyron and Burbrink 2009)
	VIM_Ex6_R2	CAA TAT CAA GAG CCA TCT TTA CAT T	

Table 1 – Description of the primers used for the regions amplified.

The PCR mix for *Cytb* was performed in a 20 µL volume, containing 4 µL of 5x HOT FIREPol® Blend Master Mix Ready to Load with 10 mM $MgCl_2$, 0.5 µL of each primer and approximately 50 ng of DNA. For *DNAH3*, *PRLR* and *SPTBN1*, the PCR mix was performed in a 25 µL volume, containing 5 µL of 5x Green GoTaq® Flexi Buffer, 3 mM of $MgCl_2$, 0.05 mM of each dNTP, 0.5 µL of each primer, 0.1 µL of GoTaq and approximately 50 ng of DNA. Finally, the PCR mix for *VIM* was performed in a 25 µL volume, containing 5 µL of 5x Green GoTaq® Flexi Buffer, 1.5 mM of $MgCl_2$, 0.05 mM of each dNTP, 0.5 µL of each primer, 0.2 µL of Boverine Serin Albumine (BSA), 0.1 µL of GoTaq and approximately 50 ng of DNA. In all PCR's, a negative control was run which included every reagent except the DNA to test for contaminations.

PCR cycles were performed in Biometra TProfessional ThermoCyclers and an Applied Biosystems Veriti Thermal Cycler. For *Cytb*, the PCR cycling was initiated by a pre-denaturing step of 15 minutes at 95ºC, followed by 35 cycles of a denaturing step of 45 seconds at 95ºC, a primer annealing step of 45 seconds at 48ºC and an extension step of 1 minute and 30 seconds at 72ºC, followed by a final extension step of 10 minutes at 72ºC. PCR cycling for *DNAH3* was initiated by a pre-denaturing step of 5 minutes at 94ºC, followed by 35 cycles of a denaturing step of 30 seconds at 94ºC, an annealing step of 40 seconds at 51.5ºC and an extension step of 1 minute and 20 seconds at 72ºC, followed by a final extension step of 10 minutes at 72ºC. As for *PRLR* and *SPTBN1*, PCR

cycling was initiated by a pre-denaturing step of 5 minutes at 94ºC, followed by 35 cycles of a denaturing step of 35 seconds at 94ºC, an annealing step of 45 seconds at 54ºC, for *PRLR*, or 51.5ºC, for *SPTBN1*, and an extension step of 1 minute and 30 seconds at 72ºC, followed by a final extension step of 10 minutes at 72ºC. Finally, PCR cycling for *VIM* was initiated by a pre-denaturing step of 5 minutes at 94ºC, followed by 35 cycles of a denaturing step of 30 seconds at 94ºC, an annealing step of 45 seconds at 52.5ºC and an extension step of 1 minute and 30 seconds at 72ºC, followed by a final extension step of 10 minutes at 72ºC.

In order to examine the results of the reactions, 2 µL of each PCR sample were run in 2% agarose gel with GelRed Nucleic Acid Stain, together with the ladder M5, and then checked in an ultraviolet transilluminator. In the end, the PCR product of the samples with positive results was sent to the GENEWIZ company (United Kingdom) along with their respective primers so they could be purified and sequenced by Sanger methods.

Sequence data and analyses

The sequences obtained were firstly blasted in the NCBI database of GenBank (Benson et al. 2013) to confirm their species identity. After that, chromatographs were revised manually, assembled, aligned using MUSCLE algorithm (Edgar 2004) and edited in Geneious v. 4.8.5 (www.geneious.com; Kearse et al. 2012). Additionally, in the nuclear genes (*DNAH3*, *PRLR*, *SPTBN1* and *VIM*), polymorphic sites were identified, and coded according to the IUPAC code, based on the Geneious plugin “find heterozygotes”, setting a peak similarity of 50%. Then, they were manually checked. Finally, the coding genes (*Cytb*, *DNAH3* and *PRLR*) were translated and subjected to a search for STOP codons.

The haplotype phase of the nuclear fragments was determined using the PHASE algorithm (Stephens et al. 2001) implemented in DNAsp v. 5 (Librado and Rozas 2009). The phases were estimated with 1000 iterations, 1 as thinning interval and 1000 burn-in iterations, under a model with recombination (-MR0 option) and 0.5 of output threshold probability (so that all phases were resolved in the final alignments). The PHASE method shows good performances in haplotype reconstructions (Garrick et al. 2010) and allows the inference of haplotypes, avoiding cloning procedures and reducing the requirements of time and resources in the lab.

For all the nuclear fragments recombination detection was performed in RDP v. 4 (Martin et al. 2015) using three different algorithms: RDP (Martin and Rybicki 2000), GENECONV (Padidam et al. 1999), and MaxChi (Smith 1992), with default options.

Table 2 – Details on the samples used in the analyses: sampling locality code, sample's name in database, taxonomic identification made by the collector in the field, coordinates of collection and genes amplified and sequenced. 'X' stands for an available sequence and '-' stands for a missing sequence.

LOCALITY CODE	SAMPLE	TAX ID	LAT	LONG	Cytb	DNAH3	PRLR	SPTBN1	VIM
1	G10804	*H. algirus intermedius*	20.310	-12.976	X	X	X	X	X
2	G654	*H. algirus intermedius*	20.557	-12.633	X	X	X	X	X
3	Z5783	*H. algirus*	21.526	-12.864	X	X	X	X	X
4	G10805	*H. algirus intermedius*	22.208	-16.524	X	X	X	X	X
5	Z13634	*H. algirus*	22.609	-12.557	X	X	X	X	X
6	Z13637	*H. algirus*	22.614	-12.567	X	X	X	X	X
7	DB19977	*H. algirus*	27.835	-12.884	X	X	X	X	X
8	Z12222	*H. algirus*	28.285	-11.201	X	X	X	X	X
9	G6242	*H. algirus intermedius*	28.468	-11.178	X	X	X	X	X
10	G6247	*H. algirus intermedius*	28.528	-10.947	X	-	X	-	-
11	Z8400	*H. hippocrepis*	28.536	-10.946	X	X	X	X	X
12	F13472	*H. hippocrepis*	28.353	-10.868	X	X	X	X	X
13	Z10610	*H. algirus*	28.606	-10.847	X	X	X	X	X
14	G6245	*H. algirus intermedius*	28.628	-10.820	X	X	X	X	X
15	F13475	*H. algirus*	28.441	-10.566	X	X	X	X	X
16	Z9051	*H. hippocrepis*	28.716	-10.519	X	X	X	X	X
17	F13476	*H. algirus*	28.508	-10.443	X	X	X	X	X
18	F13459	*H. hippocrepis*	29.005	-10.383	X	X	X	X	X
19	Z9050	*H. hippocrepis*	28.882	-10.193	X	X	X	X	X
20	F13406	*H. algirus*	28.520	-9.907	X	X	X	X	X
21	G6244	*H. algirus intermedius*	29.083	-9.904	X	X	X	X	X
22	DB12168	*H. hippocrepis*	29.087	-9.898	X	X	X	X	X
23	G6243	*H. algirus intermedius*	29.174	-9.714	X	X	X	X	X
24	MNNA	*H.* intermediate form	28.520	-9.540	X	X	X	X	X
25	Z10598	*H. hippocrepis*	29.661	-9.504	X	X	X	X	X
26	Z7194	*H. algirus*	28.391	-9.491	X	X	X	X	X
27	G11064	*H. algirus intermedius*	29.071	-9.431	X	X	X	X	X
28	G13136	*H. algirus intermedius*	29.242	-9.187	X	X	X	X	X
29	G13137	*H. algirus intermedius*	29.242	-9.187	X	X	X	X	X
30	Z9002	*H. algirus*	29.729	-8.479	X	X	X	X	X
31	M123	*H.* intermediate form	30.120	-8.410	-	-	X	X	X
32	DB13763	*H. algirus*	29.723	-7.973	X	X	X	X	X
33	G8104	*H. algirus intermedius*	29.734	-7.973	X	X	X	X	X
34	DB23847	*H. hippocrepis*	30.956	-7.817	X	X	X	X	X
35	F13349	*H. algirus*	30.190	-7.812	X	X	X	X	X
36	Z5384	*H. hippocrepis*	31.026	-7.759	X	X	X	X	X
37	DB11012	*H. hippocrepis*	30.808	-7.584	X	X	X	X	X
38	Z7034	*H. hippocrepis*	30.803	-7.530	X	X	X	X	X
39	G6246	*H.* intermediate form	30.835	-7.003	X	X	X	X	X
40	DB79	*H. hippocrepis*	32.489	-5.931	X	X	X	X	X
41	DB135	*H. hippocrepis*	32.461	-5.785	X	X	X	X	X

(continuation of Table 2)

42	Z7015	*H. hippocrepis*	32.790	-5.326	X	X	X	X	X
43	DB1525	*H. hippocrepis*	32.262	-5.147	X	X	X	X	X
44	DB1569	*H. hippocrepis*	32.262	-5.147	X	X	X	X	X
45	DB1562	*H. hippocrepis*	32.222	-4.677	X	X	X	X	X
46	M149	*H. algirus*	31.410	-4.280	X	X	X	X	X
47	Z6961	*H. hippocrepis*	32.995	-4.132	X	X	X	X	X
48	M127	*H. hippocrepis*	34.360	-2.360	X	X	X	X	X
49	M117	*H. hippocrepis*	33.120	-2.020	X	X	X	X	X
50	G8094	*H. algirus intermedius*	32.547	-1.955	X	X	X	X	X
51	Z8996	*H. algirus*	32.199	-1.612	X	X	X	X	X
52	G8503	*H. algirus intermedius*	32.162	-1.590	X	X	X	X	X
53	M112	*H. algirus*	32.090	-1.240	-	-	X	-	X
54	Z8338	*H. algirus*	32.542	-1.121	X	X	X	X	X
55	G9488	*H. hippocrepis*	32.768	0.553	X	X	X	X	X
56	G9487	*H. hippocrepis*	33.793	0.747	X	X	X	X	X
57	G714	*H. algirus intermedius*	33.569	1.164	X	X	X	X	X
58	G7911	*H. algirus intermedius*	34.109	2.999	X	X	X	X	X
59	Z917	*H. algirus*	35.053	6.166	X	X	X	X	X
60	Z922	*H. hippocrepis*	35.405	6.395	X	X	X	X	X
61	G9629	*H. algirus intermedius*	34.939	6.931	X	X	X	X	X
62	DB7	*H. hippocrepis*	36.465	8.740	X	X	X	X	X
63	DB5236	*H. hippocrepis*	36.291	8.787	X	X	X	X	X
64	DB1	*H. hippocrepis*	36.546	9.414	X	X	X	X	X
65	G8842	*H. algirus algirus*	33.539	9.989	X	X	X	X	X
-	G7054	*H. nummifer*	-	-	X				
-	G7860	*H. ravergieri*	-	-	X				
-	G7861	*H. ravergieri*	-	-	X				
-	G7863	*H. ravergieri*	-	-	X				
-	G10548	*H. ravergieri*	-	-	X				

Phylogenetic tree analyses

The *Cytb* fragment was submitted to an analysis in PartitionFinder v. 1.1.1 (Lanfear et al. 2012) to choose the most appropriate partitioning scheme and to select the most appropriate substitution model for each partition. The analysis was performed following the configurations: linked branch lengths; set of models for RAxML (for the RAxML analysis), MrBayes (for the MrBayes analysis) and BEAST (for the BEAST analysis); AIC criterion of model selection (for the RAxML analysis) and BIC (for the Bayesian analyses: MrBayes and Beast); and all schemes search.

The phylogenetic analyses with the *Cytb* fragment were performed using maximum likelihood (ML) and Bayesian (BI) methods using *Hemorrhois nummifer* and *Hemorrhois ravergieri* sequences as outgroups. The maximum likelihood analyses were

performed using RAxML (Stamatakis 2006) graphical front-end, raxmlGUI v. 1.5b2 (Silvestro and Michalak 2012) under the specific substitution models estimated in the PartitionFinder analysis. ML searches were carried out with 100 random additional replicates and support for nodes was assessed through 1000 bootstrap iterations (Felsenstein 1981).

The Bayesian analyses were performed in MrBayes v. 3.2.6 (Ronquist et al. 2012) and BEAST v. 1.8.1 (Drummond et al. 2014). In MrBayes, the partitions and evolution model configurations for each partition were implemented, according to the results of the PartitionFinder analysis. The analysis was performed with two runs, 10 million MCMC generations, with a sampling frequency of 1000 generations. The final consensus tree was calculated after a 25% burn-in. In BEAST, the substitution models across partitions were unlinked and then the model estimated by PartitionFinder for each codon position was defined. A Coalescent model of evolution was applied with constant population size (Kingman 1982) and for the estimation of divergence times a relaxed clock model with mean divergence rate of 0.0134 (stdev: 0.0021) per million years was implemented. The substitution rate used was calculated by Daza et al. (2009) for Colubrid snakes. CIPRES science gateway (Miller et al. 2010) was used to perform the two BEAST runs. These runs had a total of 30 million MCMC generations each, sampling parameters every 3000 generations. After the runs, Tracer v. 1.6 (Rambaut et al. 2013) was used to assess the posterior trace plots and the effective sample size values (ESS) of the parameters, which were greater than 200. All runs were then combined with LogCombiner v. 1.8.1 with a 25 % of burn-in and the Maximum Clade Credibility Tree was calculated with TreeAnnotator v. 1.8.1 (both the programs are available in the BEAST package).

After all ML and BI analyses, FigTree v. 1.4.2 (Rambaut 2014) was used to view the phylogenetic trees and export related graphics. The nodes were analysed and considered strongly supported when ML bootstrap values (BV) ≥ 80% and posterior probability (PP) support values ≥ 0.95. Furthermore, the mean *p*-distances with pairwise deletion were calculated for the *Cytb* fragments, using MEGA 7 (Kumar et al. 2016).

Phylogenetic network analyses

The phylogenetic relationships between haplotypes were also estimated using the statistical parsimony networks (TCS model) approach, for all the fragments amplified, using TCS v. 1.21 (Clement et al. 2000). A connection limit of 95% was defined, gaps were treated as a 5th state, and the networks were checked and coloured in TCS

beautifier (Santos et al. 2015). Furthermore, QGIS v. 2.18.9 (available at http://www.qgis.org) was used to construct a map with the geographic location of the samples, which was then modified in Microsoft PowerPoint 2016 to assess the geographical distribution of the haplotypes and clades.

Results

Dataset

The final multiple sequence alignments had 669 bp (base pairs) for *Cytb*, 680 bp for *DNAH3*, 504 bp for *PRLR*, 857 bp for *SPTBN1* and 608 bp for *VIM*. The final datasets showed no missing sequences for *PRLR*, one for *VIM*, two for *Cytb* and *SPTBN1* and three missing sequences for *DNAH3*. The total dataset had less than 3% of missing sequences, with 322 sequences obtained and 8 failed sequences (Table 2).

The RDP analyses indicate no evidence of recombination in nuclear gene alignments ($p>0.05$).

Phylogenetic analyses of mitochondrial data

The results of the PartitionFinder analyses for the best partition scheme and substitution models are presented in Table 3.

Both MrBayes and RAxML phylogenetic analyses of *Cytb* haplotypes showed two major monophyletic clades, highly supported both by posterior probabilities (PP) and bootstrap values (BV). The first clade (A) includes mostly individuals identified as *H. algirus*, the second clade (B) includes mainly *H. hippocrepis* individuals (Figure 9). A total of nine individuals classified in the field as *H. hippocrepis* appear included in the *H. algirus* clade and two other individuals classified as *H. algirus intermedius* appear included in the *H. hippocrepis* clade. Also, the two intermediate individuals that have an available *Cytb* sequence were both included in the *H. algirus* clade.

The mean *p*-distance calculated between these two clades is 11.1%, whereas intra-clade mean genetic distance was 2% and 1.9% within the *H. algirus* and *H. hippocrepis* clades, respectively (Table 4). Within the *H. algirus* clade, there is a subclade composed by the 4 easternmost samples (subclade A5), also highly supported by PP and BV values, that forms a basal clade to the remaining individuals. This subclade is composed by the only individual identified as *H. algirus algirus*, two individuals identified as *H. algirus intermedius* and one identified just as *H. algirus*. It also presents a mean genetic divergence of 6.7% from the remaining samples of the *H. algirus* clade (Table 5).

	MrBayes	RAxML	BEAST
1st	K80+I	GTR+G	K80+I
2nd	HKY	GTR+G	HKY
3rd	GTR	GTR+G	TrN

Table 3 – Results of the PartitionFinder analyses.

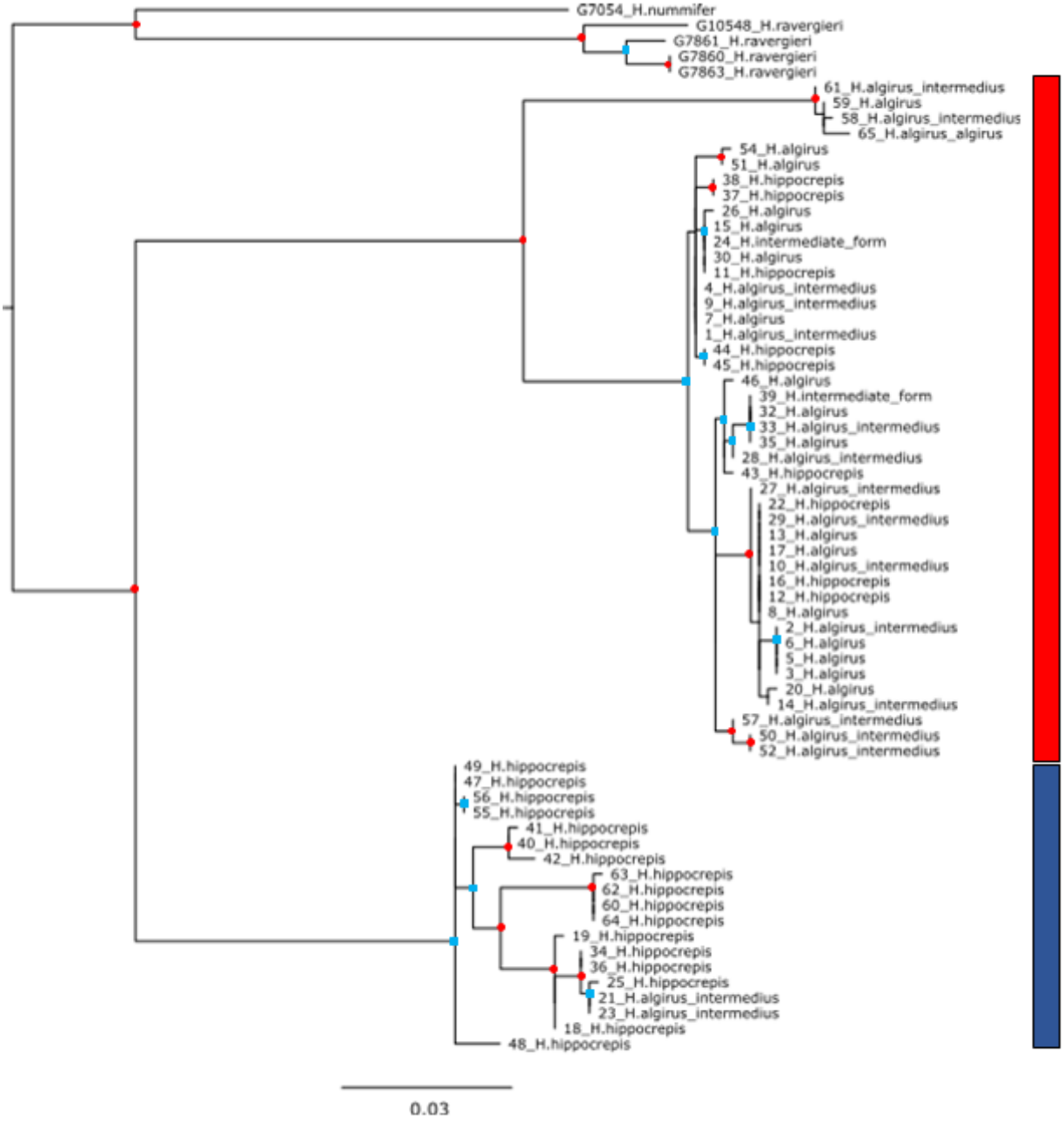

Figure 9 – ML phylogenetic tree of mtDNA (*Cytb* fragment) showing the genetic relationships between and within the *Hemorrhois algirus* and *Hemorrhois hippocrepis* individuals analysed. *Hemorrhois nummifer* and *Hemorrhois ravergieri* were used as outgroups. The nodes supported by the MrBayes analysis are also shown. Red dots are placed in nodes with ≥ 95% PP (form MrBayes analysis) and ≥ 80 BV (from RAxML analysis) support and blue squares are placed in nodes with only ≥ 95% PP. Coloured bars are representing the two major clades correspondent to *H. algirus* (A; in red) and *H. hippocrepis* (B; in blue).

	Clade A	Clade B
Clade A	**0.020**	-
Clade B	0.111	**0.019**

Table 4 – Mean genetic *p*-distances calculated between mitochondrial clades (down-left), and within them (bold values). Clade A and B represent the H. algirus and H. hippocrepis clades, respectively.

	Subclade A5
Other A subclades	0.067

Table 5 – Mean genetic *p*-distances calculated between the subclade A5 and the remaining A subclades.

The network analysis of the *Cytb* fragment, represented in figure 10, shows some genetic structure between and within the *H*. *algirus* and *H*. *hippocrepis* clades. In both clades, easternmost individuals formed distinct and well supported subclades. The structuring of the network also allows the grouping of some individuals forming other subclades that appear to be less divergent. These subclades are also present in both MrBayes and RAxML analyses, which also are concordant in their phylogenetic relationships. Nevertheless, Bayesian analyses provide higher support than ML analysis for these subclades.

The geographical distribution of the *Cytb* clades and subclades inferred in the previous analyses show a northern distribution of the *H. hippocrepis* clade and a southern distribution of the *H. algirus* one (Figure 11). All subclades have an allopatric distribution, with the exception of the subclade A4. Indeed, the subclade A4 presents a wide geographic distribution including the ranges of the subclades A1, A2 and A3. These *H. algirus* subclades are present in the western part of the species range, while the subclade A5 occupies the eastern part that was sampled.

The BEAST analysis estimated that the divergence of both *Hemorrhois algirus* and *H. hippocrepis* with the clade formed by the other two members of the genus, *H. nummifer* and *H. ravergieri* occurred about 7.7 Million years ago (Ma; 95% High Posterior Density interval, 95%HPD: 4.89-10.89 Ma). The divergence between the *H. algirus* and the *H. hippocrepis* clades occurred around 6 Ma (95%HPD: 3.65-8.65), which is approximately at the same time of the divergence between *H. nummifer* and *H. ravergieri* (5.2 Ma; 95%HPD: 2.49-7.81 Ma). The diversification within the *H. algirus* and the *H. hippocrepis* clades started around 2.9 Ma (95%HPD: 1.62-4.43 Ma) and 1.3 Ma (95%HPD: 0.67-2 Ma), respectively. Nevertheless, the *H. hippocrepis* samples used are not well distributed along its range, since it was not the purpose of the study. As for the diversification of the western clades, which may represent the *H. a. intermedius* subspecies, it was estimated to have occurred around 0.7 Ma (95% HPD: 0.38-1.17 Ma). The *H. a. algirus* clade is only represented by 4 samples, therefore estimate of diversification time for this subclade are not presented. The BEAST analysis results are presented on figure 12.

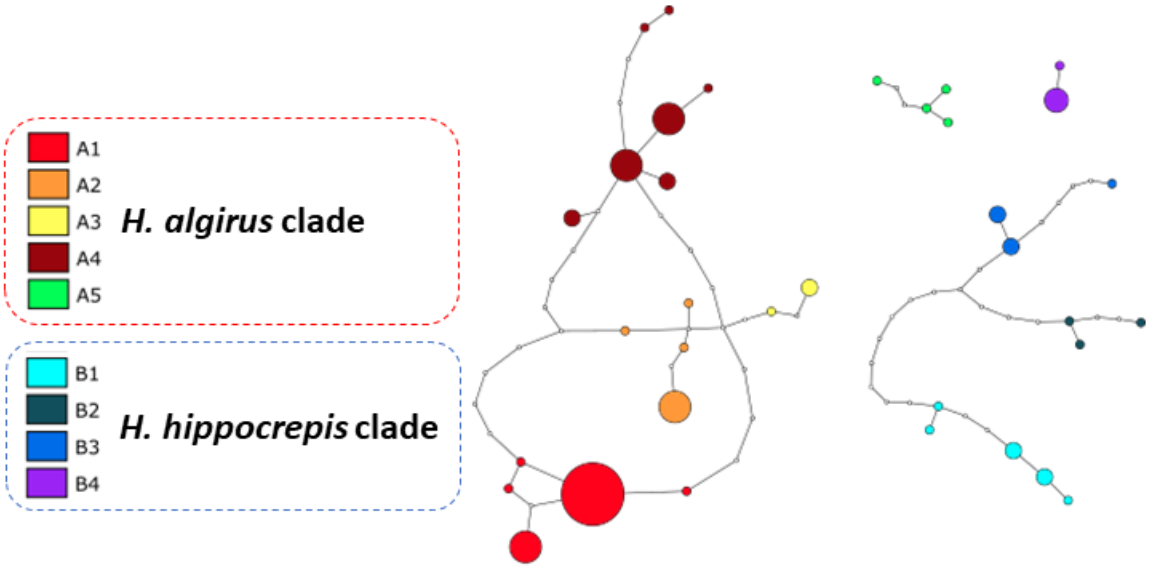

Figure 10 – Mitochondrial (Cytb) haplotype networks based on TCS model approach. The colours represent distinct subclades within each main clade (*H. algirus* or *H. hippocrepis*).

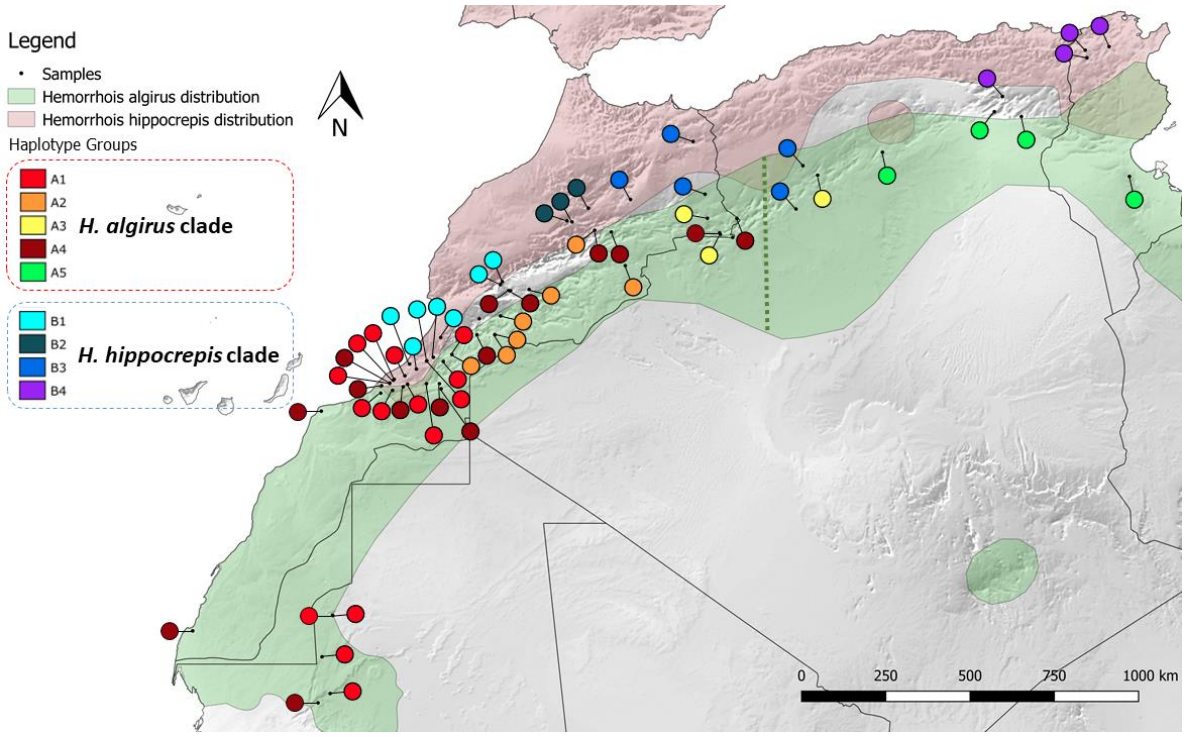

Figure 11 – Map showing the geographical distribution of the different mitochondrial (Cytb) clades and subclades inferred in the phylogenetic and network analyses. Colours represent different subclades within each main clade (*H. algirus* or *H. hippocrepis*). The distributions of *H. algirus* and *H. hippocrepis* are represented in pale green and pale pink, respectively (Miras et al. 2009; Wagner and Wilms 2013). The green dotted line represents the separation between *H. algirus algirus*, distributed to the east, and *H. algirus intermedius*, distributed to the west, according to Schätti (1986).

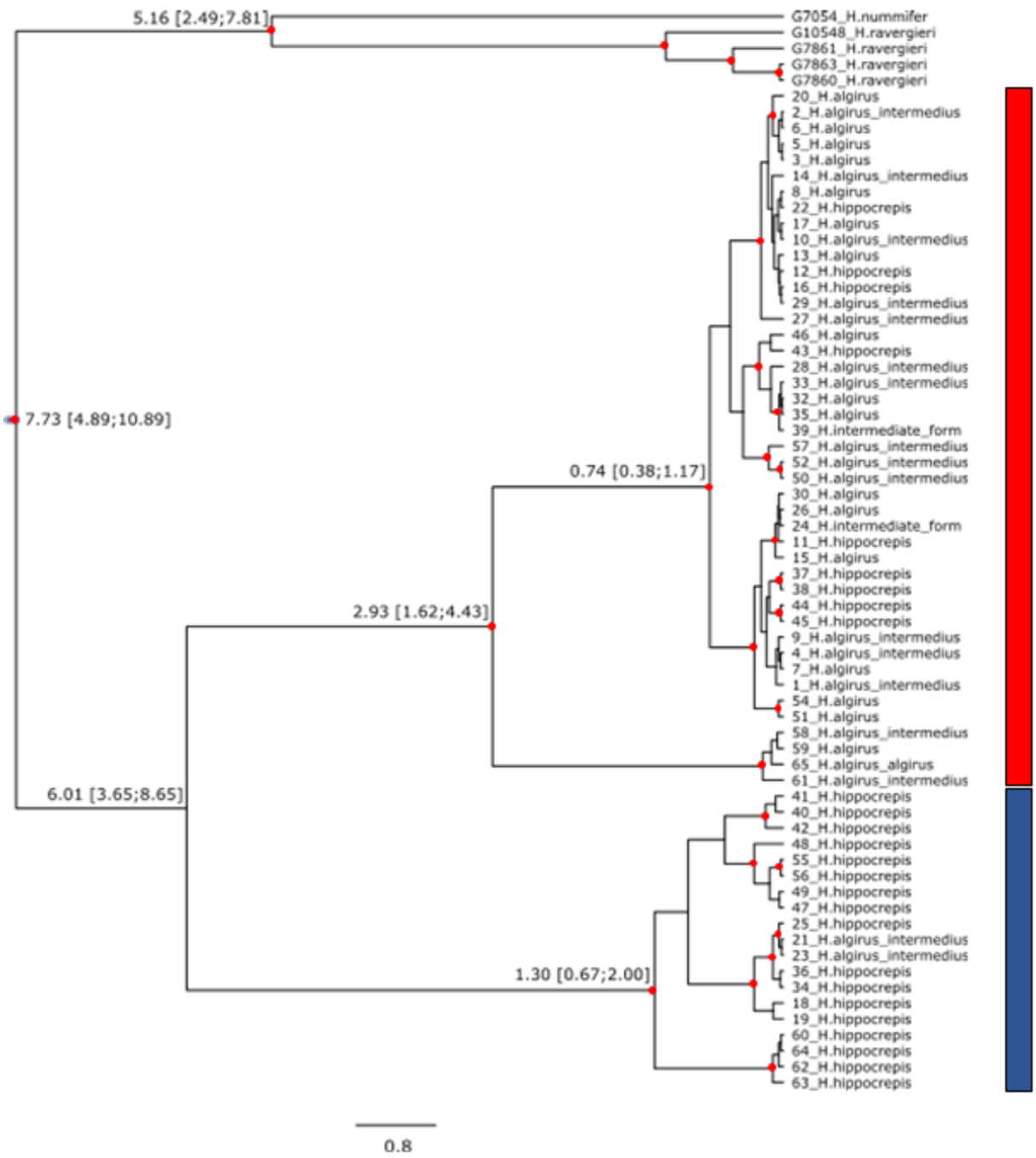

Figure 12 – BEAST maximum clade credibility tree of the *H. algirus* and *H. hippocrepis* individuals sampled. Divergence times correspond to the mean posterior estimate of their age in million years and the values within square parenthesis represent the 95% HPD (highest posterior density) interval. The red dots in the nodes indicate nodes with posterior probability (PP) values ≥ 95%. The coloured bars represent the two genetic clades, representing *H. algirus* (red bar) and *H. hippocrepis* (blue bar).

Phylogenetic analyses of nuclear data

The network analyses of both *DNAH3* and *PRLR*, show very little phylogenetic structure (Figures 13 and 16) with no apparent partition in haplotype clades. Both nuclear genes show very high levels of haplotype sharing between individuals that belong to different mitochondrial clades (Figures 14 and 17). The geographical distribution of the haplotypes of both genes (Figures 15 and 18) also shows a weak spatial structuring, with perhaps the exception of the four easternmost samples of each clade. Excepting for one *PRLR* haplotype, these specimens present, for each clade, an exclusive group of haplotypes not shared with the other individuals sampled.

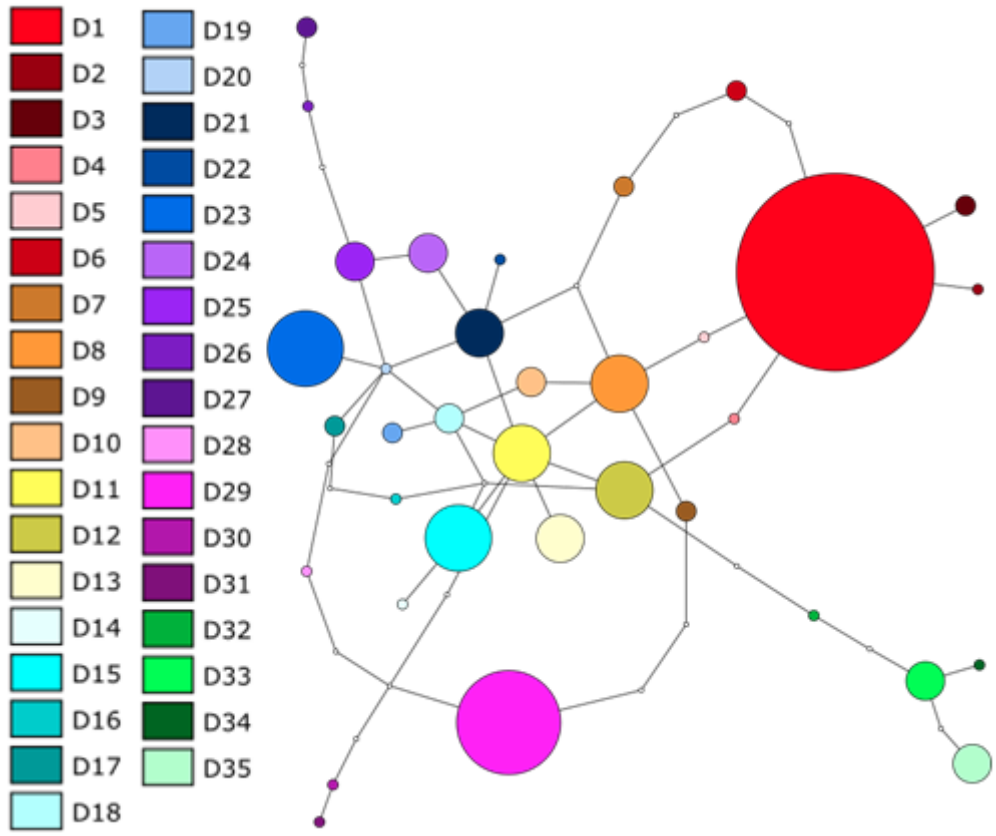

Figure 13 - *DNAH3* haplotype network based on TCS model approach. Each colour represents one haplotype.

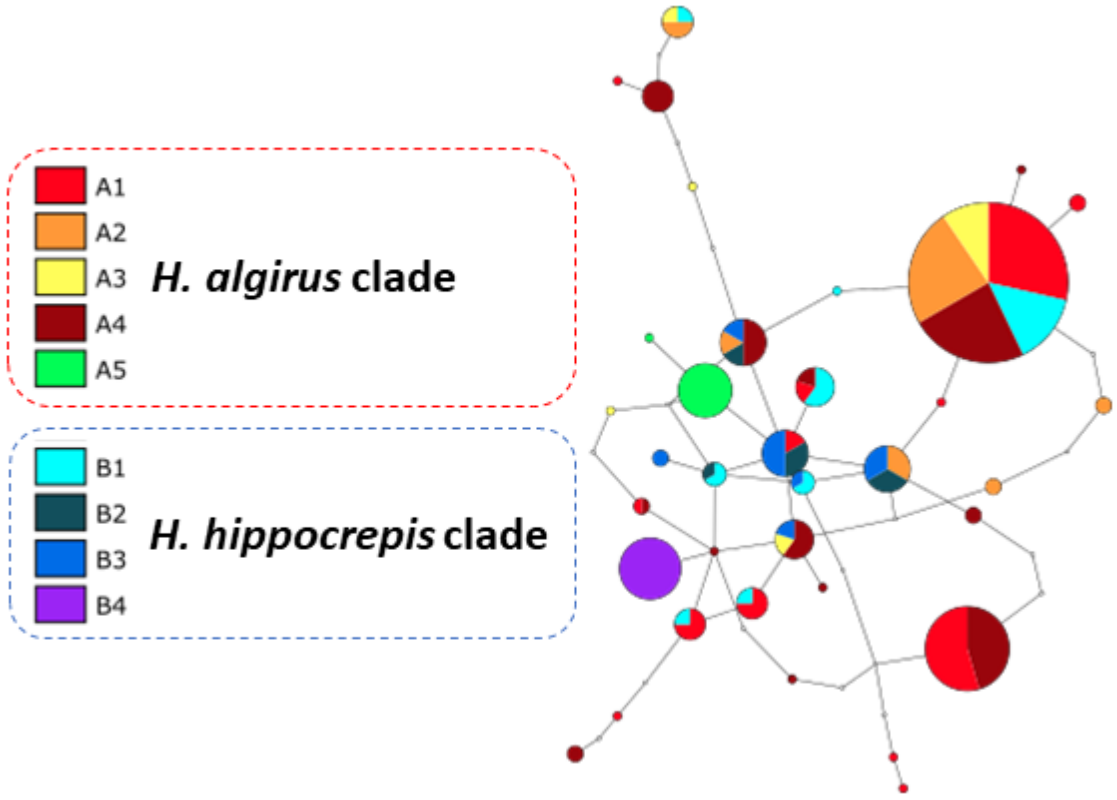

Figure 14 – *DNAH3* haplotype network based on TCS model approach. The colours represent distinct subclades within each main mitochondrial (*Cytb*) clade (*H. algirus* or *H. hippocrepis*).

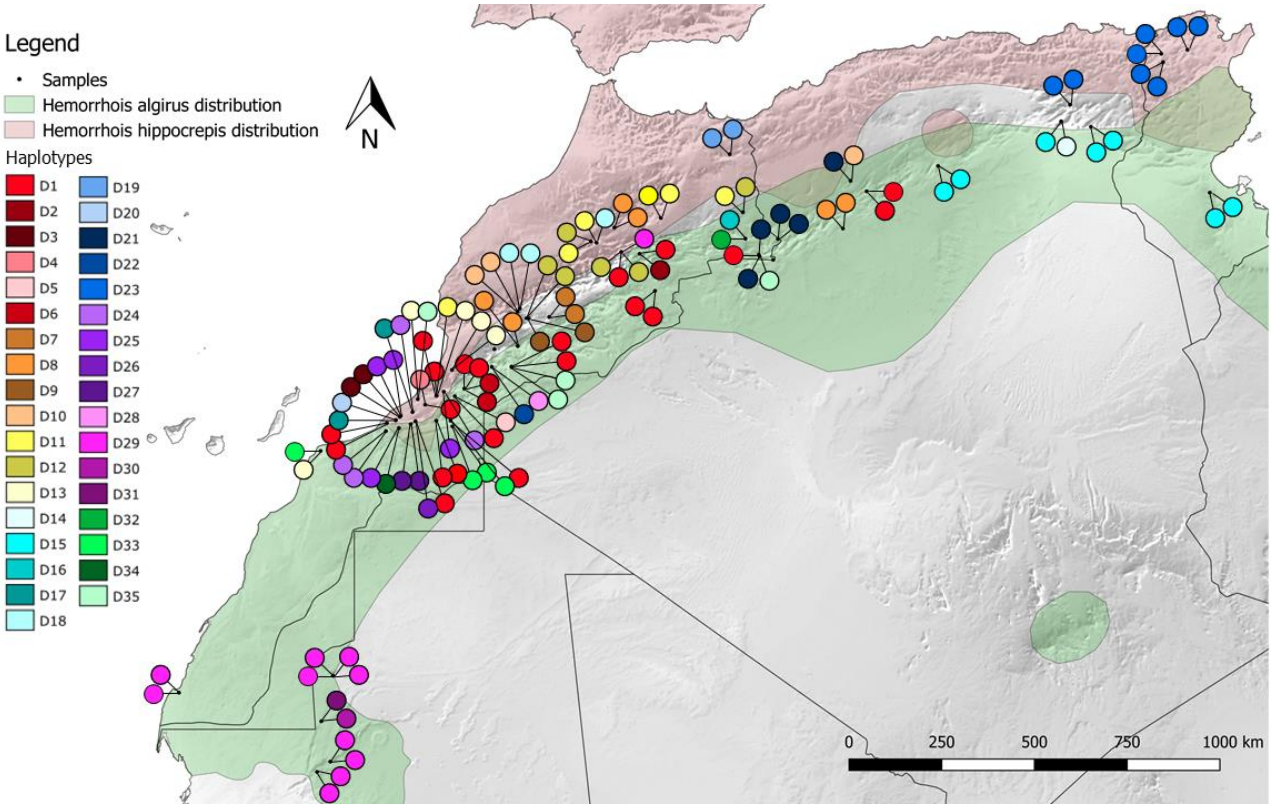

Figure 15 – Map showing the geographical distribution of the *DNAH3* haplotypes. Each colour represents one haplotype. The distributions of *H. algirus* and *H. hippocrepis* are represented in pale green and pale pink, respectively (Miras et al. 2009; Wagner and Wilms 2013).

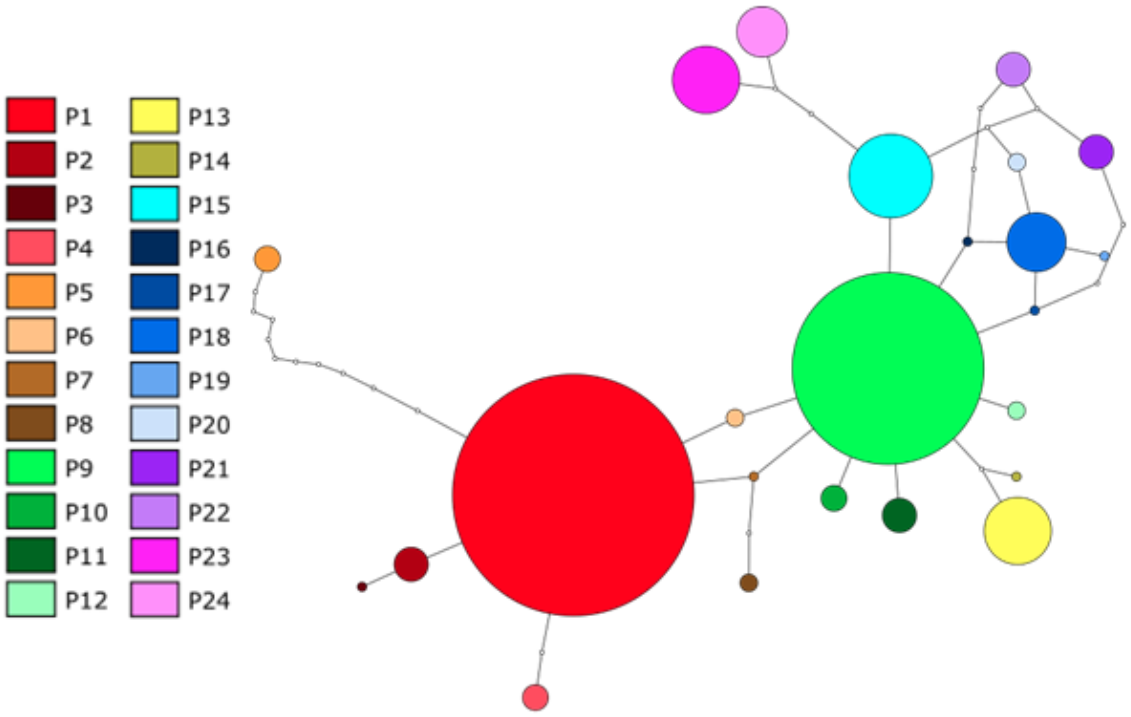

Figure 16 – *PRLR* haplotype network based on TCS model approach. Each colour represents one haplotype.

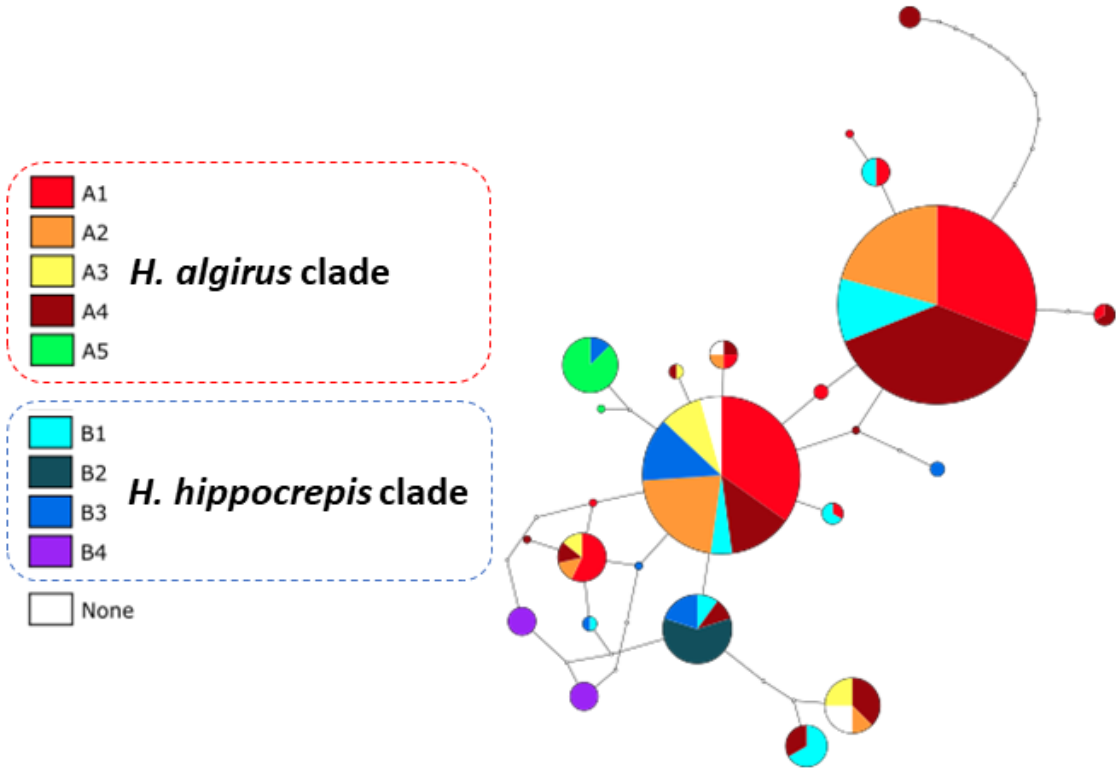

Figure 17 – *PRLR* haplotype network based on TCS model approach. The colours represent distinct subclades within each main mitochondrial (*Cytb*) clade (*H. algirus* or *H. hippocrepis*).

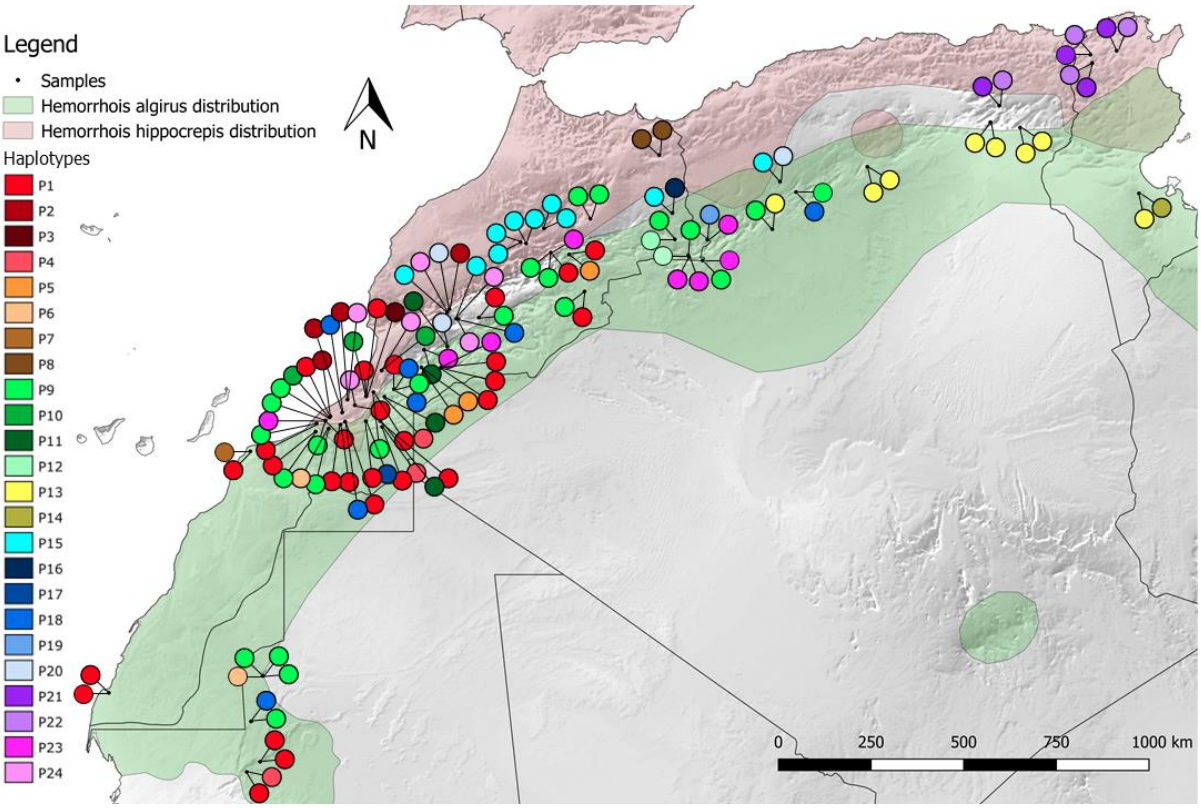

Figure 18 – Map showing the geographical distribution of the *PRLR* haplotypes. Each colour represents one haplotype. The distributions of *H. algirus* and *H. hippocrepis* are represented in pale green and pale pink, respectively (Miras et al. 2009; Wagner and Wilms 2013).

Regarding the nuclear non-coding genes analysed, the network analyses already show some degree of structuring, with two main haplotypes representing mostly individuals of each clade (Figures 19 and 22). While *SPTBN1* shows some degree of haplotype sharing between individuals of different mitochondrial clades, in *VIM* the haplotype sharing between mitochondrial clades is very low and happens just with a few individuals in the two major haplotypes (Figures 20 and 23). The geographical distribution of haplotypes of both the *SPTBN1* and *VIM* genes shows a similar structure to that observed with the mitochondrial haplotypes, with one major haplotype group covering a northern position and the other having a southern distribution. Figures 21 and 24 show that this north-south partition is more clear in *VIM* than in *SPTBN1*. Furthermore, the four easternmost individuals that belong to the *H. algirus* clade also present an exclusive set of haplotypes, not shared with any of the remaining samples.

Table 6 presents a list of the haplotypes identified in all genes for each specimen.

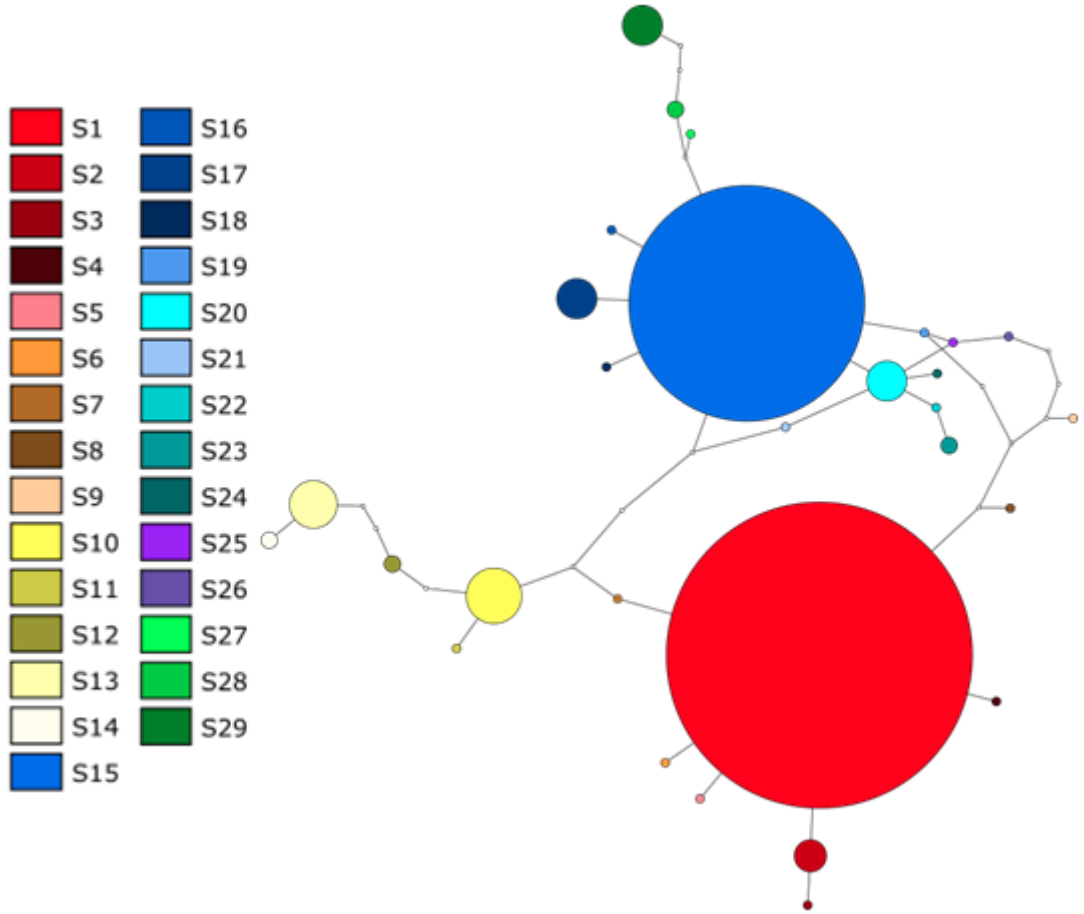

Figure 19 – *SPTBN1* haplotype network based on TCS model approach. Each colour represents one haplotype.

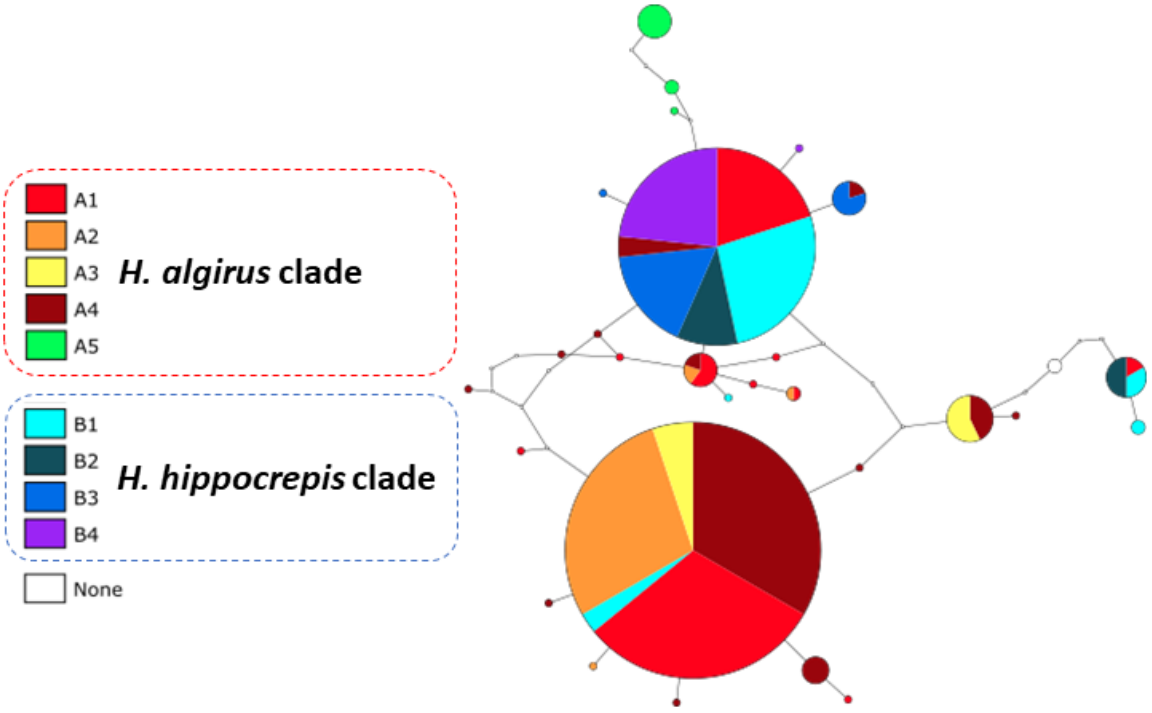

Figure 20 – *SPTBN1* haplotype network based on TCS model approach. The colours represent distinct subclades within each main mitochondrial (*Cytb*) clade (*H. algirus* or *H. hippocrepis*).

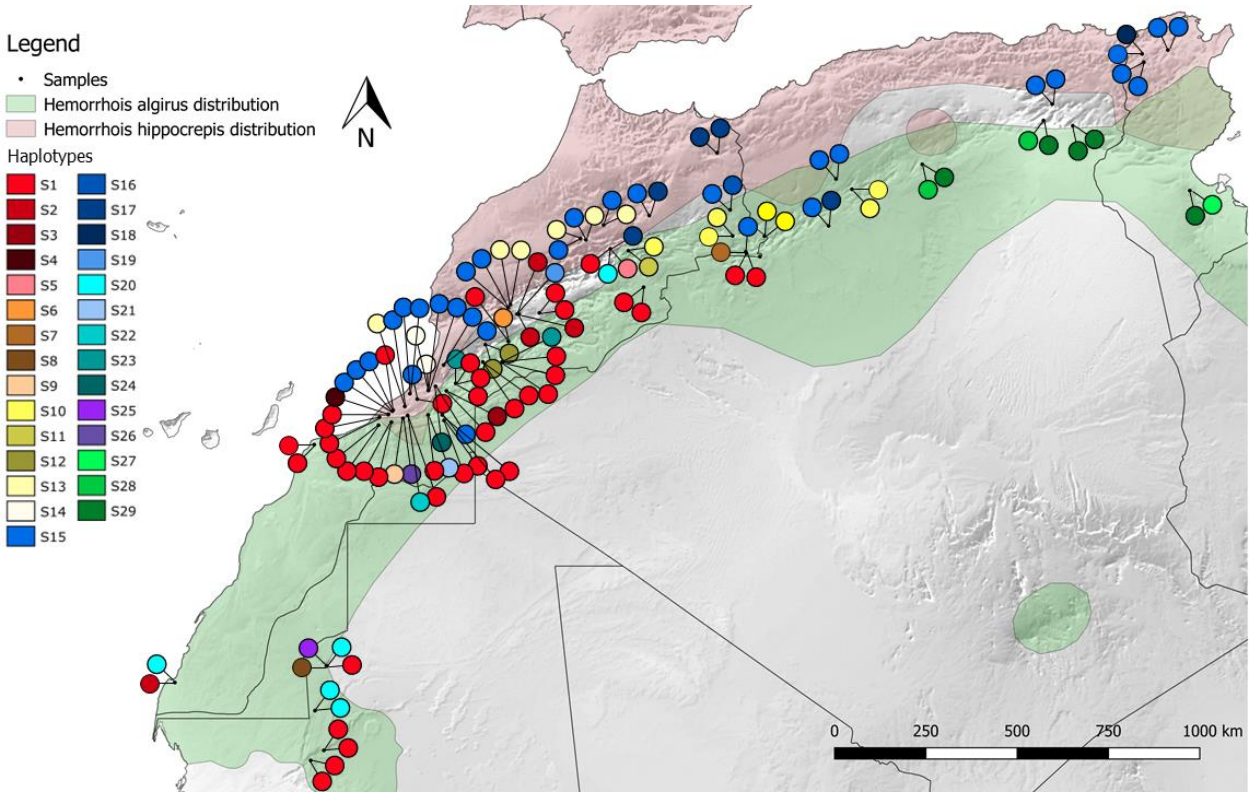

Figure 21 – Map showing the geographical distribution of the *SPTBN1* haplotypes. Each colour represents one haplotype. The distributions of *H. algirus* and *H. hippocrepis* are represented in pale green and pale pink, respectively (Miras et al. 2009; Wagner and Wilms 2013).

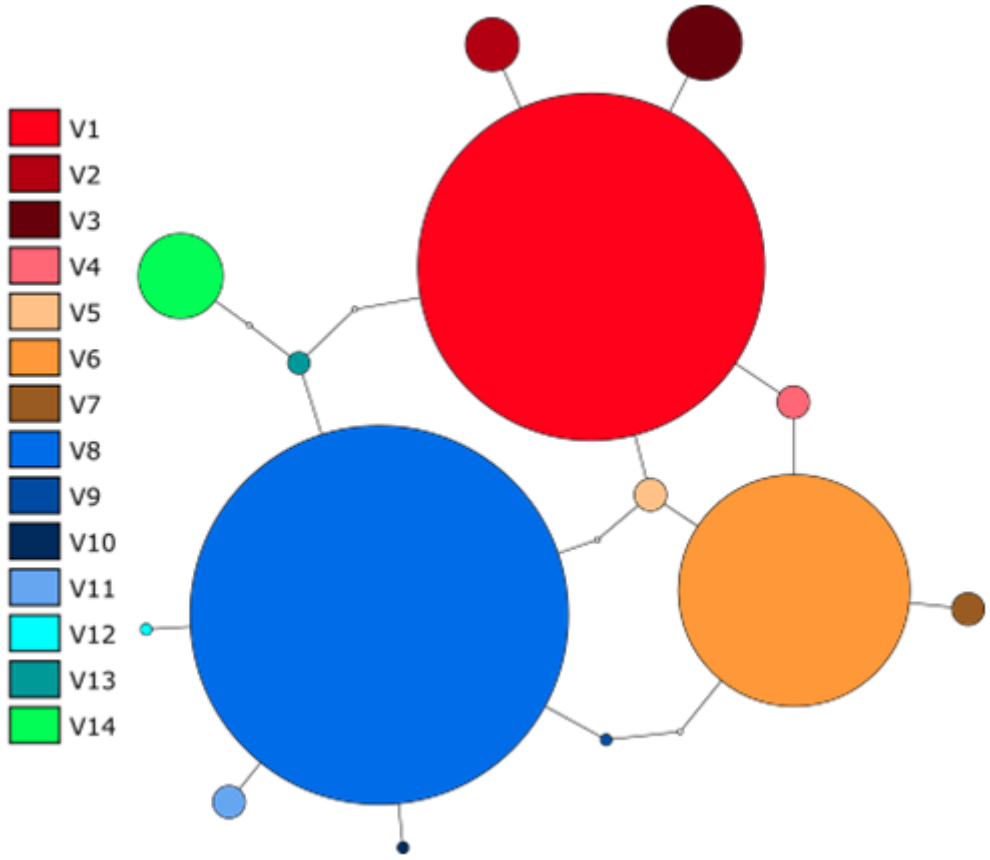

Figure 22 – *VIM* haplotype network based on TCS model approach. Each colour represents one haplotype.

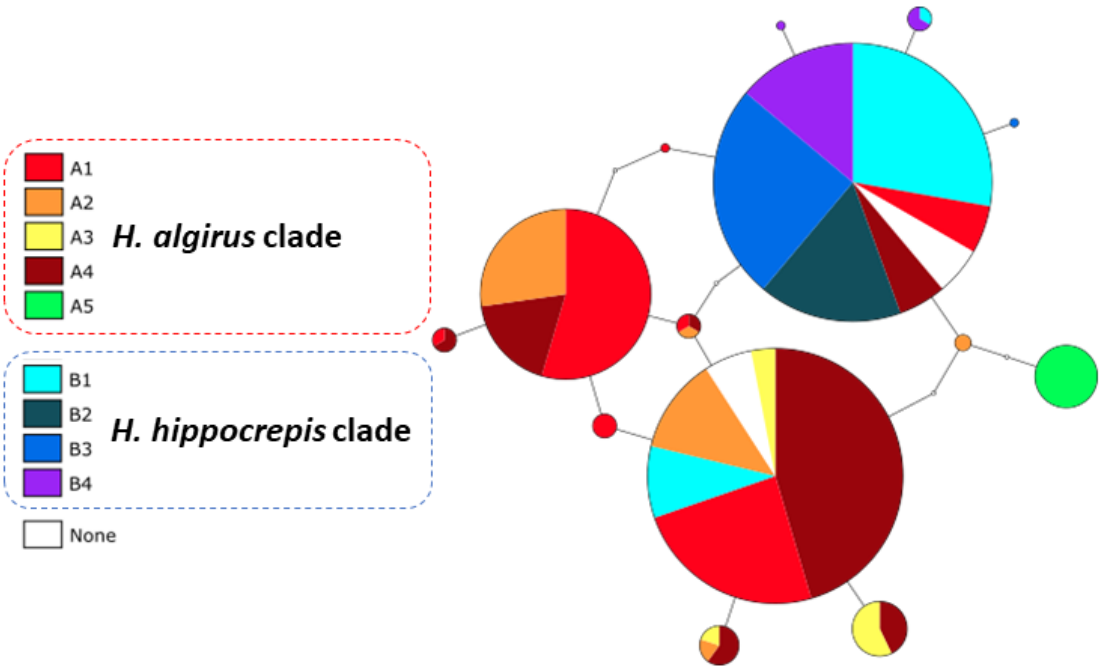

Figure 23 – *VIM* haplotype network based on TCS model approach. The colours represent distinct subclades within each main mitochondrial (*Cytb*) clade (*H. algirus* or *H. hippocrepis*).

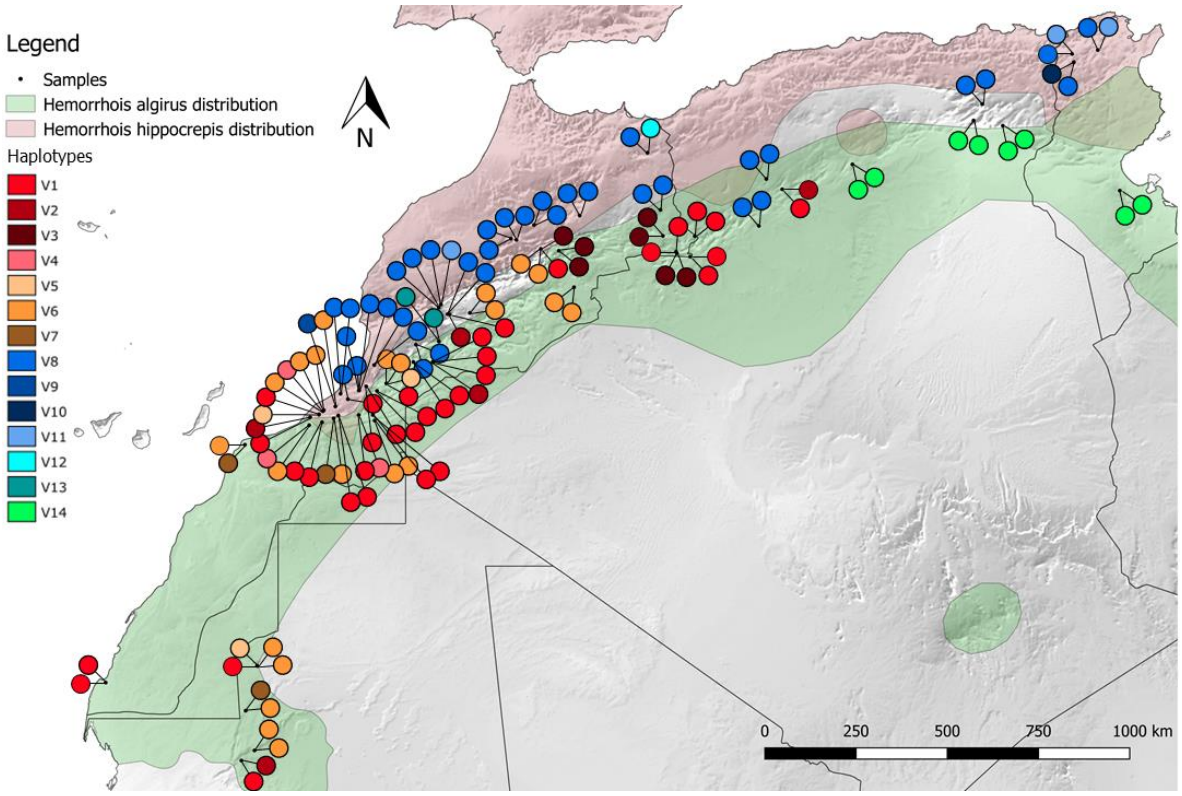

Figure 24 – Map showing the geographical distribution of the *VIM* haplotypes. Each colour represents one haplotype. The distributions of *H. algirus* and *H. hippocrepis* are represented in pale green and pale pink, respectively (Miras et al. 2009; Wagner and Wilms 2013).

Table 6 – List of the haplotypes inferred for each sample.

CODE	Haplotypes								
	Cytb	DNAH3		PRLR		SPTBN1		VIM	
1	C12	D29	D29	P1	P4	S1	S1	V1	V2
2	C4	D29	D29	P1	P1	S1	S1	V6	V6
3	C4	D30	D31	P9	P18	S20	S20	V6	V7
4	C12	D29	D29	P1	P1	S2	S20	V1	V1
5	C4	D29	D29	P9	P9	S1	S20	V6	V6
6	C4	D29	D29	P6	P9	S8	S25	V1	V5
7	C12	D13	D33	P1	P7	S1	S1	V6	V7
8	C1	D24	D24	P1	P9	S1	S1	V4	V6
9	C12	D1	D1	P1	P9	S1	S1	V1	V2
10	C1	-	-	P1	P9	-	-	-	-
11	C15	D17	D20	P9	P21	S1	S4	V1	V5
12	C1	D25	D34	P6	P9	S1	S1	V1	V1
13	C1	D3	D3	P9	P10	S15	S15	V4	V6
14	C2	D25	D25	P1	P2	S1	S15	V6	V6
15	C15	D27	D27	P1	P1	S9	S26	V6	V7
16	C1	D17	D24	P2	P18	S13	S15	V6	V9
17	C1	D1	D26	P1	P18	S1	S22	V1	V1
18	C26	D13	D35	P2	P24	S15	S15	V8	V8
19	C27	D1	D4	P1	P24	S1	S15	V1	V8
20	C3	D1	D1	P1	P17	S1	S20	V1	V4
21	C23	D1	D1	P1	P10	S14	S14	V8	V8
22	C1	D11	D13	P1	P3	S15	S15	V8	V8
23	C23	D24	D25	P1	P9	S15	S24	V1	V1
24	C15	D1	D33	P1	P11	S1	S1	V1	V1
25	C24	D13	D13	P10	P24	S15	S15	V8	V8
26	C16	D33	D33	P1	P4	S1	S1	V6	V6
27	C5	D1	D5	P4	P11	S1	S3	V1	V1
28	C7	D6	D6	P9	P18	S1	S1	V1	V5
29	C1	D1	D1	P1	P18	S1	S23	V6	V6
30	C15	D22	D28	P5	P5	S1	S1	V1	V1
31	-	-	-	P11	P21	S12	S12	V8	V8
32	C6	D35	D35	P1	P1	S1	S1	V1	V2
33	C6	D1	D1	P1	P21	S1	S23	V1	V1
34	C25	D10	D10	P15	P24	S15	S15	V8	V8
35	C6	D8	D8	P1	P11	S1	S6	V13	V13
36	C25	D18	D18	P2	P20	S13	S13	V8	V11
37	C14	D12	D12	P15	P24	S2	S19	V8	V8
38	C14	D9	D9	P18	P24	S2	S2	V1	V2
39	C6	D7	D7	P1	P9	S1	S1	V6	V6
40	C29	D11	D12	P15	P15	S13	S15	V8	V8
41	C30	D11	D18	P15	P15	S13	S15	V8	V8
42	C28	D8	D8	P15	P15	S13	S15	V8	V8
43	C8	D1	D12	P9	P9	S1	S20	V6	V6

(Continuation of Table 6)

44	C13	D12	D29	P1	P21	S5	S17	V1	V3
45	C13	D1	D2	P1	P5	S10	S11	V3	V3
46	C9	D1	D1	P1	P9	S1	S1	V6	V6
47	C31	D11	D11	P9	P9	S15	S17	V8	V8
48	C33	D19	D19	P8	P8	S17	S17	V8	V12
49	C31	D11	D12	P15	P16	S15	S16	V8	V8
50	C11	D16	D32	P9	P12	S10	S10	V3	V3
51	C17	D1	D21	P9	P12	S7	S15	V1	V1
52	C11	D21	D35	P21	P21	S1	S1	V3	V3
53	-	-	-	P9	P21	-	-	V1	V1
54	C18	D21	D21	P19	P21	S10	S10	V1	V1
55	C32	D8	D8	P9	P13	S15	S17	V8	V8
56	C32	D10	D21	P15	P20	S15	S15	V8	V8
57	C10	D1	D1	P9	P18	S10	S10	V1	V2
58	C19	D15	D15	P13	P13	S28	S29	V14	V14
59	C20	D14	D15	P13	P13	S28	S29	V14	V14
60	C34	D23	D23	P21	P22	S15	S15	V8	V8
61	C21	D15	D15	P13	P13	S29	S29	V14	V14
62	C34	D23	D23	P21	P22	S15	S15	V8	V10
63	C35	D23	D23	P21	P22	S15	S18	V8	V11
64	C34	D23	D23	P21	P22	S15	S15	V8	V11
65	C22	D15	D15	P13	P14	S27	S29	V14	V14

Discussion

Phylogeny and taxonomy of *Hemorrhois algirus*

The results of the mitochondrial phylogenetic analyses are consistent with the current, most widely accepted taxonomy of the genus *Hemorrhois*. Two main phylogenetic clades are found within this genus: one with the Asian members *H. nummifer* and *H. ravergieri* and another with the African members *H. algirus* and *H. hippocrepis*. The two African species appear well differentiated from each other. The network analysis and the geographical distribution of the haplotypes also corroborate these results, with *H. hippocrepis* haplotypes found in the northern region and *H. algirus* haplotypes bordering them by the south, as was expected by the species distribution present in their IUCN Red List webpages (Miras et al. 2009; Wagner and Wilms 2013). However, these distribution maps need some updating, as some individuals were found outside the ranges defined by the IUCN assessors of these species.

Also, the distribution of the two main haplotype groups of *H. algirus* correspond rather well to the expected distribution of the subspecies *H. algirus algirus* and *H. algirus intermedius* reported by Schätti (1986). Two individuals from Central and North-eastern Algeria were identified as *H. algirus intermedius* and clustered in the *H. algirus algirus* group (58 and 61 in the map of Fig. 7). These individuals were more than 250 km East form the expected subspecies border reported by Schätti (1986), deep into the putative *H. a. algirus* range, which suggests these individuals were misidentified. Nevertheless, the morphological differences between these subspecies should be reassessed. Further studies should incorporate more samples from this border area, in Central and North-western Algeria, to try to infer if there is a contact zone between these two subspecies and the actual morphological differences between them. However, it is true that sampling efforts in this area are very difficult to implement, due to the political instability of this country. This has contributed for the poor knowledge about this phylogeographical break, that is also present in many other organisms such as for example *Macroprotodon* snakes, *Uromastyx* lizards and *Buthus* scorpions (Carranza et al. 2004; Wilms et al. 2007; Pedroso et al. 2013).

The fact that the Mauritanian and the south Western Saharan individuals belong to the same mitochondrial haplotype clades of individuals from southern Morocco indicate that populations from these regions have not been separated for too long. Also, it confirms the synonymy of *Hemorrhois* (*Coluber*) *algirus villiersi* Bons, 1962, and *H. algirus intermedius* as proposed by Schätti (1986).

On the other hand, the easternmost individuals of *H. algirus,* which represent the *H. algirus algirus* subspecies, form a monophyletic unit which is well distinct from all western individuals in each of the analysed loci. Mitochondrial phylogenetic analyses indicate that this subclade has an old divergence from the *H. algirus intermedius* subclades (around 2.9 Ma). Further studies sampling the whole geographic range of *H. a. algirus* are necessary to draw taxonomic conclusion on the possible full species rank of this subspecies which would imply the nomenclatural change of *H. a. intermedius* as *Hemorrhois intermedius* Werner, 1929.

Evolutionary history of *Hemorrhois algirus*

The time of divergence between the African and Asian species of *Hemorrhois* was estimated to be around 7.7 Ma based on mitochondrial data. This period coincides with the first appearance of the Saharan desert (7 Ma; Le Houerou 1997; Pound et al. 2012). It is assumed that the ancestors of these geographic groups were previously distributed continuously along the southern Mediterranean coast and they were then separated by an advancing zone of aridity (Nagy et al. 2004).

The estimated time of divergence between *H. algirus* and *H. hippocrepis* was 6 Ma, which is in agreement with the estimated divergence time of about 4-7 Ma between these two species obtained by Carranza et al. (2006). This period corresponds to the transition from the Miocene to the Pliocene, just after the first appearance of the Saharan desert, around 7 Ma (Le Houerou 1997; Pound et al. 2012). In fact, this period also corresponds to the second and biggest event of uprising of the Atlas mountains, which continues today (Babault et al. 2008; de Lamotte et al. 2008; Robert-Charrue and Burkhard 2008; de Lamotte et al. 2009). The mountains could have promoted a barrier that stimulated the speciation event and the adaptation of *H. algirus* to more arid environments. Speciation in other colubrid snakes from North Africa of the *Macroprotodon* species complex is also estimated to have occurred in the same period (Carranza et al. 2004).

Within the *Hemorrhois algirus* clade, the separation of the two subspecies was estimated to have occurred around 2.9 Ma. This coincides with the onset of glaciation cycles with the alternations between wet/hot and dry/cold periods, which were associated to the contraction and expansion of arid habitats in North Africa (DeMenocal 1995; DeMenocal 2004; Jacobs et al. 2010). Being a semi-arid adapted species, *H. algirus* populations must have had to flee the extreme desert conditions caused by a Saharan expansion during the glacial periods, by moving northwards to more temperate and

coastal areas and maybe even up the Atlas Mountains, in those regions where these mountains would have limited northward movements. This scenario has been recently proposed by Brito et al. 2014 as common in the evolutionary histories of Sahara-Sahel species. These movements must have led the populations to experience periods of isolation during the glacial phases, leading to the divergence between subclades. Indeed, most of the subclades within *H. algirus intermedius* show an allopatric distribution. Their time of coalescence is around 740,000 years ago, suggesting that are likely the result of isolation into distinct refugia during glacial periods at the beginning of the Middle Pleistocene. On the other hand, we do not observe multiple lineages within *H. algirus algirus*. However, the low sample size of *H. a. algirus* does not allow a comprehensive assessment, thus further studies should better sample within the range of this subspecies.

Relationships between *H. algirus* and *H. hippocrepis*

The fact that some individuals were identified in the field (based on phenotypic characters) as one species and then were found to have mitochondrial (*Cytb*) haplotypes correspondent to the other species, raises some questions about past or present hybridizations between the two species. All these individuals are restricted to three areas in Morocco: the Tan-Tan-Guelmin area, the Ouarzazate area and the northwest Errachidia area. Since *H. algirus* is not recognized to be present in high mountains (Bons and Geniez 1996), these areas could be separated by the Anti-Atlas and the High Atlas mountain chains. The Tan-Tan-Guelmin and Ouarzazate areas are also previously recognized as contact zones between *H. algirus* and *H. hippocrepis* and areas where can be found intermediate individuals (Bons and Geniez 1996; Marín and Barroso 2012). From two individuals with available pictures (22 and 37; Figure 25), we can confirm that some of the specimens classified as *H. hippocrepis* but possessing *H. algirus* mitochondrial haplotypes have indeed a phenotypic pattern characteristic of *H. hippocrepis*, with a horseshoe mark and rounded spots along the body. However, the coloration of these individuals is rather unusual, since individual 22 has a greenish brown base colour and individual 37 has a brown pattern that is usually darker in *H. hippocrepis*. This follows the pattern of some of the intermediate individuals already reported by Marín and Barroso (2012). The individual 24 was classified as an intermediate form because despite having morphological characteristics of *H. hippocrepis*, it was found in a habitat where *H. algirus* might have been expected, as reported by its collector. This individual was confirmed to have a *H. algirus* mitochondrial haplotype. As for the two individuals

Figure 25 – Pictures of the individuals 22 and 37, respectively. Location and credits from left to right: Bouizakarne, Morocco, photo by Pedro Sousa; Tizi Melloul, Morocco, photo by Miguel Carretero.

identified as *H. algirus* but having a *H. hippocrepis* mitochondrial haplotype (21 and 23) there are no photos available, but it could be important to assess some information concerning their morphology.

It is worth bearing in mind that the morphological traits used for species identification are most likely polygenic, thus, intermediate morphotypes between species likely underlies a pattern of extensive introgression shared among several nuclear loci. In this light, we can obtain further information on putative hybrid zones between these species from the nuclear genetic data we obtained.

Nuclear loci show two distinct patterns of variation. On one hand, the networks of *VIM* and *SPTBN1* present a phylogeographic structure consistent with mitochondrial data, with two major groups corresponding to the two species and a few instances of mismatch between mitochondrial and nuclear genetic background, i.e. individuals carrying mitochondrial haplotype typical of one species but nuclear haplotypes typical of the other species. On the other hand, the *DNAH3* and *PRLR* networks show no detectable structure and an extensive haplotype sharing between individuals belonging to distinct species and mitochondrial lineages. These results establish an incongruence between the mitochondrial and nuclear patterns of variation. Cases such as this are widely reported along animal nuclear and mitochondrial assessments (Toews and Brelsford 2012) and even in cases with deeply divergent mitochondrial lineages (Guicking et al. 2009; Giska et al. 2015; Salvi, Pinho, et al. 2017). Two important aspects to explain these results are the fact that nuclear DNA evolves typically much slower than mitochondrial DNA and the possibility of introgression between *H. algirus* and *H. hippocrepis*. In other words, we could explain the observed pattern of nuclear haplotype sharing between species, and between intraspecific mitochondrial lineages, as the result of incomplete lineage sorting at *DNAH3* and *PRLR* loci or as the result of differential

introgression across multiple loci following hybridization. These two hypotheses are not mutually exclusive.

Incomplete lineage sorting is more likely at nuclear loci because of the usually slower evolutionary rate of nuclear genome and its fourfold difference in effective population size compared to mitochondrial genome (the nuclear genome is diploid and biparentally inherited, while the mitochondrial is haploidy and uniparentally inherited). Therefore, under neutral evolution, historical isolation events are quickly imprinted in mitochondrial genomes, forming genealogical clusters, whereas it takes more time for nuclear genealogies to be sorted (Moore 1995; also see Avise 2000 and Palumbi et al. 2001). This theory has already been reported in many phylogeographical studies to explain differences in patterns of variation between nuclear and mitochondrial loci (Pinho et al. 2008; Toews and Brelsford 2012; Bisconti et al. 2013; Salvi et al. 2013; Salvi et al. 2014) and even for other Colubrids (Rato et al. 2009). This hypothesis can also be corroborated if the use of nuclear markers with faster mutation rates unveils the same patterns as the mitochondrial genome (as in Pinho et al. 2007 and Rodríguez et al. 2014). Incomplete lineage sorting is a random process which has not a spatial association.

On the other hand, the exchange of haplotypes between species via hybridization and genetic introgression is not a range-wide process, but it is instead expected to be associated to parapatric contact zones. Hybridization may have different outcomes depending on the loci analysed, what is known as patterns of differential introgression across loci. This means that, following hybridization it is possible to have lots of genetic exchange in some loci, little exchange at other ones and no genetic exchange at all in many others. Differential introgression across loci may arise from processes as natural selection, genetic drift, linkage, recombination rates variation or even a combination of them (Baack and Rieseberg 2007; Harrison and Larson 2014; Harrison and Larson 2016).

Some of the nuclear loci analysed show instances of haplotype sharing between individuals belonging to different species or mitochondrial lineages and that are far away from putative contact zone between species, suggesting geographically-random process such as the incomplete lineage sorting behind the origin of this pattern. This is something seen in some of the *DNAH3* and the *PRLR* haplotypes and even in *SPTBN1* there is a major *H. hippocrepis* haplotype (S15) that is carried by individual 51, more than 100 km away from the nearest contact zone. Adding to this, the pattern unveiled by the two faster evolving gene fragments SPTBN1 and VIM is pretty similar to the mitochondrial pattern. This suggests that incongruences between the nuclear genes *DNAH3* and *PRLR* and the mitochondrial locus is due to their slower evolutionary rates and associated slower sorting.

On the other hand, the majority of the mitochondrial-nuclear mismatches and instances of haplotype sharing involve individuals at the border of species' range which indicates that also introgression plays a role in the lack of a sharp phylogeographic structure observed at the nuclear loci. For example, in VIM, the only mismatches between nuclear and mitochondrial haplotypes are from the samples 19, 22, 23 and 37. These four individuals are present in the Tan-Tan-Guelmin and Ouarzazate areas, all places where intermediate forms have been reported (Bons and Geniez 1996; Marín and Barroso 2012).

However, it is true that the great majority of the individuals of *H. hippocrepis* analysed are from these bordering zones with the *H. algirus* range. This is something that can be positively biasing the hybridization hypothesis against the incomplete lineage sorting hypothesis, since it does not allow us to infer how deep into the range of *H. hippocrepis* the haplotype sharing between the two species goes.

In summary, with the data available it seems that incomplete lineage sorting is playing the major role in the observed mito-nuclear incongruences, but it is also true that the genetic and phenotypic pattern observed in areas of sympatry between the species fits very well with that expected in case of introgressive hybridization. Further studies should expand the sampling range for both species in order to provide a compelling explanation for the observed nuclear pattern of variation of the two species.

Conclusion

Results from mitochondrial DNA analyses showed a deep divergence between an eastern subclade and the remaining *Hemorrhois algirus* individuals, dating approximately 2.9 Ma, around the onset of the glaciations. The geographic break between these two main *H. algirus* lineages is located near the one proposed by previous studies for the division between *H. algirus algirus* and *H. algirus intermedius* distributions, leading to the conclusion that the eastern lineage is representative of the *H. a. algirus* subspecies and the western lineage represents the *H. a. intermedius* subspecies. The *H. a. algirus* lineage has a private set of haplotypes, which form a monophyletic group, in all the gene fragments analysed, which leads to question if the two subspecies deserve a full species status. This would implicate the elevation of *H. a. intermedius* to the species *Hemorrhois intermedius* Werner, 1929, since *H. a. algirus* represents the nominal subspecies for *H. algirus*. However, the low sample size correspondent to *H. algirus algirus* indicates that further studies with a better geographic sampling and additional morphological data on this lineage are needed prior to any taxonomic changes.

Relatively to the morphologically intermediate forms between *H. algirus* and *H. hippocrepis*, this study found distinct patterns of variation for the different loci analysed. If the mitochondrial marker (*Cytb*) and the nuclear markers *SPTBN1* and *VIM* showed a consistent structure, associated with the species taxonomy and with little haplotype sharing by the nuclear markers, this was not the case for the nuclear markers *DNAH3* and *PRLR* which showed no clear structure and extensive haplotype sharing between individuals belonging to distinct mitochondrial lineages. The presence of some shared haplotypes in areas far away from these species' ranges borders suggest a role for the incomplete lineage sorting at the *DNAH3* and *PRLR* loci. However, there is also a concentration in the putative contact zones of (i) shared haplotypes at all nuclear markers analysed, (ii) morphologically intermediate specimens and (iii) the mismatch between some mitochondrial haplotypes and the individual's phenotypic identification. This pattern indicates a role of hybridization and introgression in shaping the individuals genetic structure of populations in these contact areas. Further studies should include more samples away from the border between the range of these two species in order to better characterize pure and hybrid forms and to assess the contribute of incomplete lineage sorting and hybridization in the formation of the phylogeographic pattern observed. Also, morphological data of *H. algirus* and *H. hippocrepis,* especially from individuals at the species' range borders, will be useful to correlate genetic and morphological patterns of variation of these species.

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